INTERACTION OF BILIRUBIN WITH NORMAL AND ALTERED HUMAN ERYTHROCYTE MEMBRANES

Abstract

HUMA RASHID

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Biotechnology of the Aligarh Muslim University
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ABSTRACT

Interaction of bilirubin (BR) with cells or cell membranes is well documented and it is commonly accepted that the toxicity of bilirubin depends on its passage across the plasma membrane and its association with the membrane lipids. However, the way in which bilirubin interacts with biological membranes is not fully understood. Erythrocytes, being simple, have been commonly used as a model system to study the interaction of bilirubin with cells or cell membranes. Further, erythrocyte-bound bilirubin has been suggested as a useful criterion for the risk of bilirubin encephalopathy in neonates.

Although a large number of data is available on the bilirubin-membrane interaction, no conclusion has been arrived at the mechanism of entry and localization of bilirubin in the bilayer membranes. It appears that more studies are required to confirm the localization of bilirubin in the erythrocyte membranes in terms of surface binding, internal binding, hydrophobic binding, etc. Though, the presence of bilirubin binding sites on biological membranes has been suggested, characterization of the individual membrane component involved in binding and the location of the binding sites on the erythrocyte membranes have not been fully worked out.

Contradictory results achieved so far regarding the mechanism of entry of bilirubin into erythrocyte membranes calls for reinvestigation of bilirubin binding properties of membrane. To unravel the mechanism of entry of bilirubin into brain cells, we have performed studies on the interaction of bilirubin with normal

and altered human erythrocyte membranes as a model. The identification and characterization of bilirubin binding receptors on erythrocyte membranes may form the basis for the development of various preventive measures against bilirubin encephalopathy.

Interaction of bilirubin with different aged group red cell membranes showed maximum binding to old erythrocyte membranes and minimum with young erythrocyte membranes and this difference corresponds negatively with sialic acid content and phospholipid content of these membranes probably due to decreasing surface charge density with age, facilitating the penetration of bilirubin into these old membranes.

The effect of pH and temperature on the binding of bilirubin to human erythrocyte membranes was studied by incubating the membranes at different pH and temperatures, and determining the amount of bound bilirubin. Binding of bilirubin to erythrocyte membranes is independent of the ionic strength of the incubation mixture however, changes in pH greatly affects membrane-bound bilirubin. At all pH values, the amount of membrane-bound bilirubin increased with the increase in bilirubin-to-albumin molar ratios (B/As), being highest at lower pH values in all cases. Further, linear increase in bound bilirubin with the increase in bilirubin concentration in incubate was observed at a constant B/A and at all pH values. However, the value of slope increased with decrease in pH suggesting more bilirubin binding to membranes at lower pH values. Increase in bilirubin binding at lower pH can be explained on the basis of increased free

bilirubin concentration as well as more conversion of bilirubin dianion to monoanion.

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Interaction of bilirubin with different types of erythrocyte membrane vesicles such as unsealed, heterogeneous, sealed and inside-out membrane vesicles prepared from human and goat erythrocytes were studied. Bilirubin binds to both the surfaces of erythrocyte membranes and also accumulates within the lipid bilayer of the erythrocyte membrane, which cannot be removed by albumin. Out of various types of membrane vesicles, in both species, unsealed membrane vesicles bound quantitatively higher amounts of bilirubin followed by heterogeneous and sealed membrane vesicles whereas inside-out membrane vesicles bound the lowest amount of bilirubin.

These differences in the amount of bound bilirubin to different membrane vesicles were correlated well with the percentage accessibility of sialic acid to neuraminidase in these membranes suggesting that bilirubin bound preferentially to the outer layer of erythrocyte membranes than the inner layer. Further, membrane vesicles prepared from human erythrocytes bound higher amounts of bilirubin than those prepared from goat erythrocytes. This can be ascribed to different phospholipid composition of these membranes.

Binding of bilirubin to human erythrocyte membranes was studied after various enzymatic treatments as well as calcium loading. Whereas phospholipase D treatment of erythrocyte membranes resulted in 23% increase in bilirubin binding, phospholipase C-treated membranes showed remarkable enhancement in bilirubin binding. Polar head groups in general and negatively charged phosphate moieties, in particular, of phospholipids of the membrane appear to inhibit a large amount of bilirubin from binding to the membranes. Neuraminidase treatment of the membranes also led to a slight increase in bilirubin binding as compared to untreated membranes. Membrane proteins and carbohydrates seem to play significant regulatory role in bilirubin binding.

However, no direct correlation was found between the increase in bilirubin binding and the amount of carbohydrate released upon tryptic digestion of membranes. Increase in bilirubin binding to trypsin-treated membranes can be ascribed to the increase in free bilirubin concentration in the incubation mixture as a result of tryptic hydrolysis of albumin by the membrane-bound tryptic activity. Calcium-loaded erythrocyte membranes also showed remarkable increase in bilirubin binding as a result of negative charge shielding and calcium-induced hydrophobic aggregation. These results suggest the inhibitory role of polar head groups of phospholipids (phosphate in particular), carbohydrate and sialic acid in the binding of bilirubin to erythrocyte membranes.

In order to study the role of membrane proteins in bilirubin binding phenomenon, selective removal of membrane proteins was carried out using various reagents, namely, ethylenediamine tetraacetic acid (EDTA), sodium

hydroxide (NaOH), 3,5-diiodosalicylic acid, lithium salt (LIS), dimethylmaleic anhydride (DMMA), sodium iodide (NaI), o-phenanthroline-cupric sulfate (CuP) and phenanthroline-cupric sulfate reduced with 2-mercaptoethanol. Effects of these treatments on the conformation and bilirubin binding properties of the membrane were studied using CD spectroscopy as well as estimation of membrane-bound bilirubin by diazotized-color reaction. Though a significant amount of protein (ranging from 23 % to 69 %) was lost from the membranes upon these treatments, only a small decrease (3-13 %) was observed in bilirubin binding, being maximum with NaOH-treated membranes.

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Date:.....

Approved:

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Saad Tayyab, Supervisor

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INTERDISCIPLINARY BIOTECHNOLOGY UNIT
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Dr. Saad Tayyab
Reader and Member
Academic Council

Certificate

I certify that the work presented in the following pages has been carried out by ***Ms. Huma Rashid*** *and that it is suitable for the award of Ph.D. degree in Biotechnology of the Aligarh Muslim University, Aligarh.*

Saad Tayyab

Saad Tayyab, Ph.D.
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Rizwan Hasan Khan
Lecturer & Co-Supervisor

DECLARATION

I hereby declare that the thesis entitled "Interaction of bilirubin with normal and altered human erythrocyte membranes", embodies the work carried out by me.

Huma Rashid

Huma Rashid
Research Scholar
Interdisciplinary Biotechnology Unit,
Aligarh Muslim University,
Aligarh-202002, India

CONTENTS

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I won't have reached this stage in life but for the perspiring, selfless and sincere efforts of my parents, Mummy and Papa and the affection of my sisters, Sana and Ima. Their unfailing love, prayers and good wishes have been a source of inspiration. I also extend my sense of requital to my in-laws, especially Ammi for being the pillar of strength throughout and whose constant assurance allowed me to work with full grandeur. I am at a loss of words in expressing my deepest gratitude, profound regards and immense love to my dear husband, Salman, for his forbearance, affection and constant endorsement which gave me the required zeal for the completion of this work.

There are numerous other names, which I am unable to pen down because of the limitation of space. I hope it is understood that while there are no limitations to feeling of gratitude, there are some in finding words to express them.

Huma Rashid

(HUMA RASHID)

To my dear husband

Salman

who deserves a special tribute

for his moral support and

affectionate understanding

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Introduction

INTRODUCTION

Heme catabolism in the reticuloendothelial cells of liver, spleen and bone marrow leads to the formation of bilirubin as the final product. Under physiological conditions, in human adult (70 Kg), 6 g of hemoglobin equivalent to $1\text{-}2\times10^8$ erythrocytes is destroyed per day producing 250-350 mg of bilirubin (Schmid & McDonagh, 1978). Approximately 3-4 mg bilirubin/Kg of body weight is produced per day in healthy adult. In infants, production of bilirubin is high (6-8 mg/Kg body weight) because of greater erythrocyte mass and shorter life span of erythrocytes (Gourley, 1997). Both proteins globin and iron, of hemoglobin are reutilized by the body, but the iron-free porphyrin portion of heme is catabolized into bilirubin IX-α. Bilirubin IX-α can be isolated from gallstone and is the most commonly found natural linear tetrapyrole. In plasma, it is firmly bound to serum albumin in a molar ratio of 1:1 and rendered non-toxic (Bratlid & Fog, 1970).

PROPERTIES OF BILIRUBIN

(i) Structure: Protoporphyrin IX, the catabolic product of hemoglobin, opens at the α-methin bridge and leads to the formation of bilirubin IX-α (Z,Z) (Figure 1, i). This is the main component of bilirubin in the body, which is neurotoxic (Brodersen, 1980). It has a polar structure with several groups capable of forming hydrogen bonds, namely two carboxyls, two pyrrol NH, two lactam-NH, two lactam carbonyls and several hydrophobic groups i.e. four methyls, two

vinyls, two ethylenes and one methylene (Figure 1, ii). Bilirubin IX-α (Z,Z) exists either as dianion or intramolecularly hydrogen-bonded bilirubin acid (as shown in Figure 1, iii & iv). Illumination of bilirubin IX-α (Z,Z) with blue light causes cis-trans isomerization (Figure 1, ii) and leads to the formation of photobilirubins, namely bilirubin IX-α (E,Z) or bilirubin IX-α (E,E) (Lightner & Park, 1977; Pedersen *et al.*, 1977) which no longer form intramolecular hydrogen bonds. Small amounts of protoporphyrin IX are opened at β position and still smaller at γ and δ (Figure 1, i) leading to the formation of various non-α isomers (IX-β, IX-γ and IX-δ) constituting 5 % of total bilirubin (Blanckart *et al.*, 1975; Brown, 1976; Blumenthal *et al.*, 1977).

(ii) Solubility: Bilirubin IX-α (Z,Z) is nearly insoluble in water at pH values below 7.0 but is quite soluble in alkaline solutions forming salts of the dianion i.e. solubility increases markedly from acidic to alkaline pH values (Brodersen, 1980). Non-α isomers, on the other hand, are more soluble in water (Overbeek *et al.*, 1955; Brodersen, 1979) even in acid solution. Poor solubility of the α-isomer in (Z,Z) configuration is due to the presence of undissociated carboxyl groups capable of forming a complete pattern of intramolecular hydrogen bonds (Figure 1, iv). At pH 7.4, ionic strength 0.15 and 37°C, solubility of pure IX-α isomer of bilirubin is found to be 7 nM, whereas it is 0.60 μM in alkaline aqueous media at pH 8.5 (Brodersen, 1979). The solubility of bilirubin in apolar solvents, such as n-hexane is less than 1 μM and generally increases with increasing solvent polarity, reaching to a value higher than 10 mM in dimethyl sulfoxide

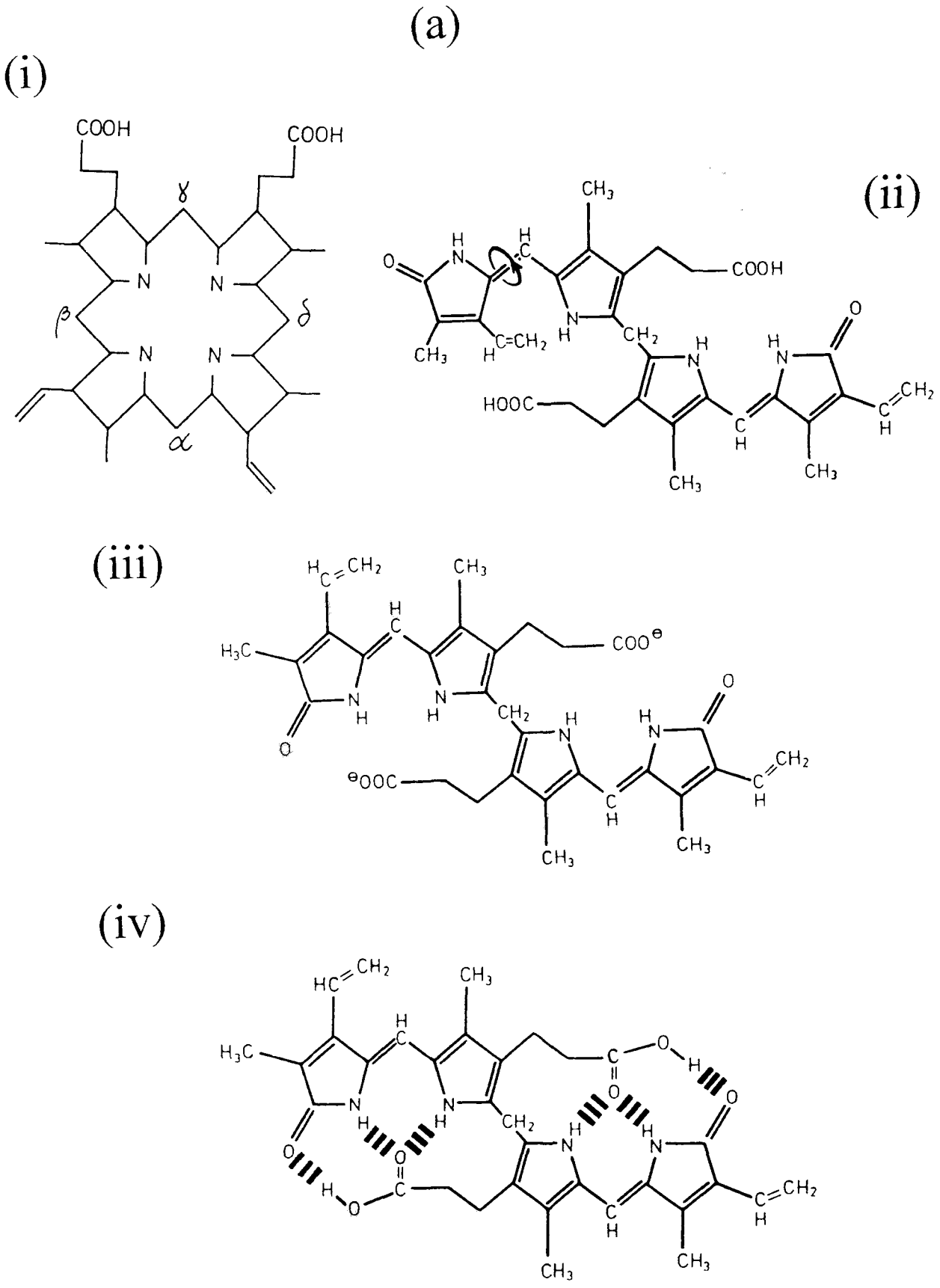
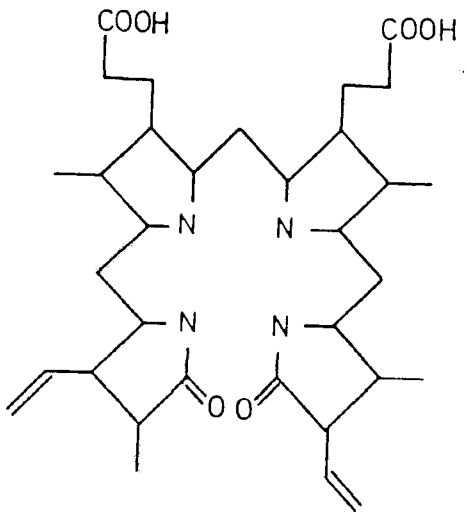


Figure 1. Chemical structure of bilirubin

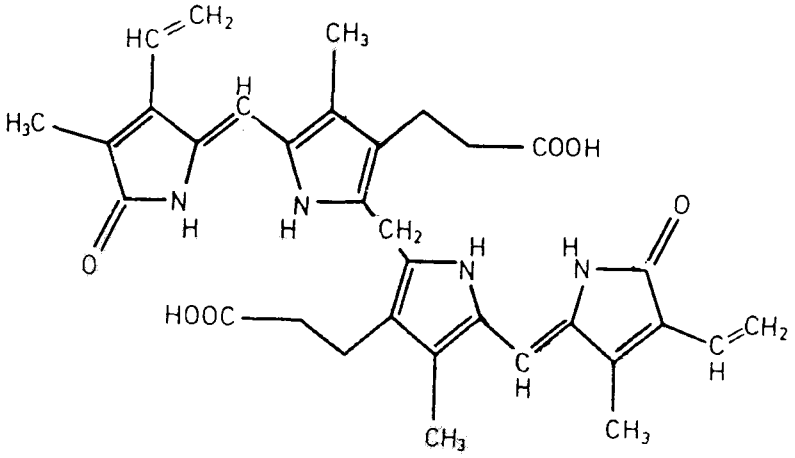
(i) Protoporphyrin IX (ii) Bilirubin IX-α (Z,Z)
(iii) BilirubinIX-α (dianion)
(iv) Bilirubin IX-α acid, intramolecularly hydrogen bonded (Adapted from Brodersen, 1979).

(b)

(i)



(ii)



(i) Bilirubin IX-α (Z,Z) (ii) Bilirubin IX-α (acid)
(Adapted from Brodersen, 1979).

(Brodersen, 1979). Earlier bilirubin was considered to be a lipophilic substance, based on the binding of bilirubin to lipid-water interphases containing lipids of a polar nature (Mustafa & King (1970). Minimum aqueous solubility at physiological pH (Ostrow *et al.*, 1994) due to formation of intramolecular hydrogen bonds (Bonnett *et al.*, 1978) can account for another speculative factor for bilirubin lipophilicity. According to its solubility characteristics, bilirubin is a polar substance and could hardly be rated as lipophilic (Brodersen, 1979). Chemical nature of bilirubin shows limited amount of lipophilic carbon hydride moieties but several strongly polar groups, i.e. two lactam and two carboxyl groups and the dianion forms a water soluble sodium salt which occurs as a monomer-dimer equilibrium mixture (Brodersen, 1966) but shows no tendency to micelle formation in alkaline solution.

(iii) Spectroscopic properties: Light absorption spectrum of bilirubin IX-α (Z,Z) acid, dissolved in organic solvents shows two maxima, one weak band at about 290 nm and another intense maximum at 450-460 nm (Heilmeyer, 1931), while spectrum of non-α isomers of bilirubin in dimethyl-formamide solution shows similar maximum although the main peak is located at shorter wavelength 390-410 nm (Blanckaert *et al.*, 1976). Bilirubin IX-α (Z,Z) acid has a molar absorption coefficient close to 60,000 $M^{-1}cm^{-1}$ at 460 nm in chloroform (Brodersen, 1979). Dilute solutions of bilirubin disodium salt in alkaline aqueous media have light absorption maximum around 430-440 nm, depending upon buffer composition and temperature (Carey & Koretsky, 1979). The molar

absorption coefficient has been reported to be in the range of 4.7-5.2×10^4 $M^{-1}cm^{-1}$ (Blauer *et al.*, 1972; Carey & Koretsky, 1979; McDonagh, 1979). The spectrum is constant with pH varying from 8-12. At low concentrations of bilirubin, from about 20 nM to an upper limit between 1-10 μM, the scattering to high values, indicates formation of large particles. The light absorption spectrum shows a slight, immediate loss of intensity followed by marked decrease of the peak at 440 nm and an increase in absorption at 500 nm with increasing time.

(iv) Bilirubin catabolism *in vivo*: Bilirubin is albumin-bound in plasma. Small amount of bilirubin is also conjugated with glucose (Blanckaert *et al.*, 1974) and xylose (Compernolle *et al.*, 1971; Fevery *et al.*, 1977). Albumin-bound bilirubin is removed from albumin in liver, taken up at the sinusoidal surface of the hepatocytes by a carrier-mediated saturable system. The smooth endoplasmic reticulum provides a set of enzymes, which convert bilirubin into diglucuronide. Similar conversion also occurs in kidney and intestinal mucosa. Secretion of this conjugated bilirubin into bile is carried out by an active transport mechanism. As the conjugated bilirubin reaches the terminal ileum and large intestine, the glucuronides are removed by specific bacterial enzymes and the pigment is reduced by the fecal flora to a group of colorless tetrapyrrolic compounds, called urobilins and finally excreted in the feces (Schmid & McDonagh, 1978; Kitamura *et al.*, 1990; Nishida *et al.*, 1992).

FUNCTIONS OF ALBUMIN

About 40 % of the albumin is found in the circulatory plasma (Peters, 1992) whereas remaining 60 % resides about half in vicera and half in muscle and skin (Rabilloud *et al.*, 1988). Albumin is reported to occur intracellularly in developing brain tissue (Pineiro *et al.*, 1982), nerve cells (Mata *et al.*, 1987) and both interstitially and intracellularly, in testis (Krishna & Spanel-Borowski, 1990). The albumin concentration in plasma in an average person is about 35-50 g/L, which declines slightly with age (Cooper & Gardner, 1989) and is lower in newborns (Cartlidge & Rutter, 1986) and as low as 20 g/L in premature infants (Reading *et al.*, 1990). Albumin performs many vital functions while circulating in blood plasma. Along with the maintenance of blood pH, it contributes ~80 % to the colloidal osmotic pressure (Figge *et al.*, 1991). It is involved in the transport of many ligands including Ca^{++} (Vorum *et al.*, 1995), long fatty acids (Brodersen & Ebbesen, 1983; Peters, 1985), thyroid hormones (Mendel *et al.*, 1990), tryptophan (Herve *et al.*, 1982; Zhang *et al.*, 1993) through reversible binding. It is also involved in the transport of pyridoxal phosphate (Fonda *et al.*, 1991), cysteine and glutathione (Joshi *et al.*, 1987) by forming covalent linkage with these ligands. The tight binding of bilirubin to albumin (Knudsen *et al.*, 1986) prevents the passage of this neurotoxic pigment into the central nervous system in newborns (Esbjorner, 1991) and helps in its delivery to the liver for conjugation and excretion. Antioxidant property of albumin-bound bilirubin has also been reported (Stocker *et al.*, 1987). Whereas bilirubin causes increase in fluidity of the erythrocyte membrane, albumin prevents the fluidity changes in

erythrocyte membranes (Chen *et al.*, 1992). The participation of albumin as the key carrier of an incredible variety of ligands serves to illustrate further the continuing recognition of the utility of albumin (Stamler *et al.*, 1992). Albumin is responsible for the transport and storehousing of many therapeutic drugs in the blood stream (Lindup, 1987).

ALBUMIN-LIGAND INTERACTION

A variety of endogenous as well as exogenous ligands bind to albumin in a reversible manner with association constant varying from 10^4-10^8 M^{-1} (Carter & Ho, 1994). Some important informations regarding the binding sites have been gained from the recently determined crystal structure of HSA (He & Carter, 1992; Sugio *et al.*, 1999). A number of selective binding sites have been shown to exist on HSA, which include drug-binding sites, I and II, as well as relatively specific sites for bilirubin and fatty acids (Figure 2, a). Although a vast majority of ligands bind to the two strong binding sites residing in subdomains II A and III A corresponding to the two sites, I and II, respectively (Sudlow *et al.*, 1976). Subdomain III A (Site II) seems to possess the primary binding activity of albumin while subdomain II A (Site I) is more specialized (Carter & Ho, 1994). The bilirubin-binding site is believed to reside in subdomain IIA (Carter & Ho, 1994). Lys-240 (Site I in subdomain II A) of HSA has been shown to be important in forming a salt bridge with carboxyl group of bilirubin (Jacobsen, 1978) and critical lysine residue(s) is(are) supposed to be buried in the protein interior (Tayyab & Qasim, 1987; Mir *et al.*, 1992). About 50 drugs have been

(a)

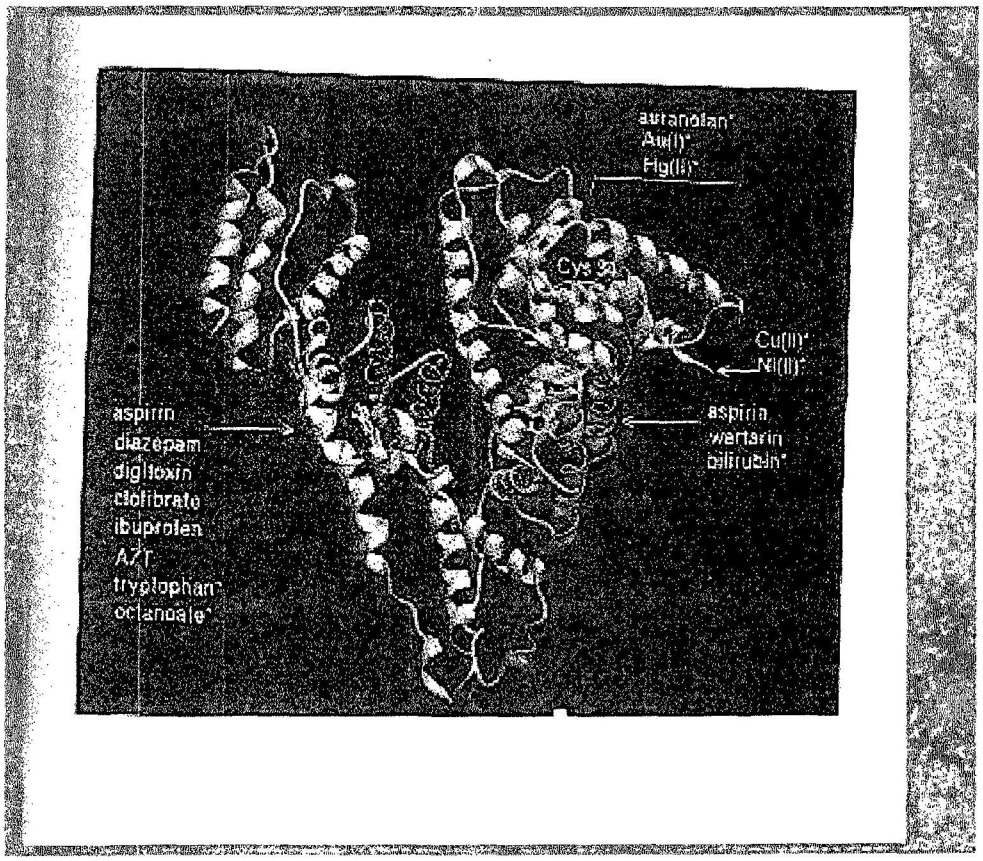
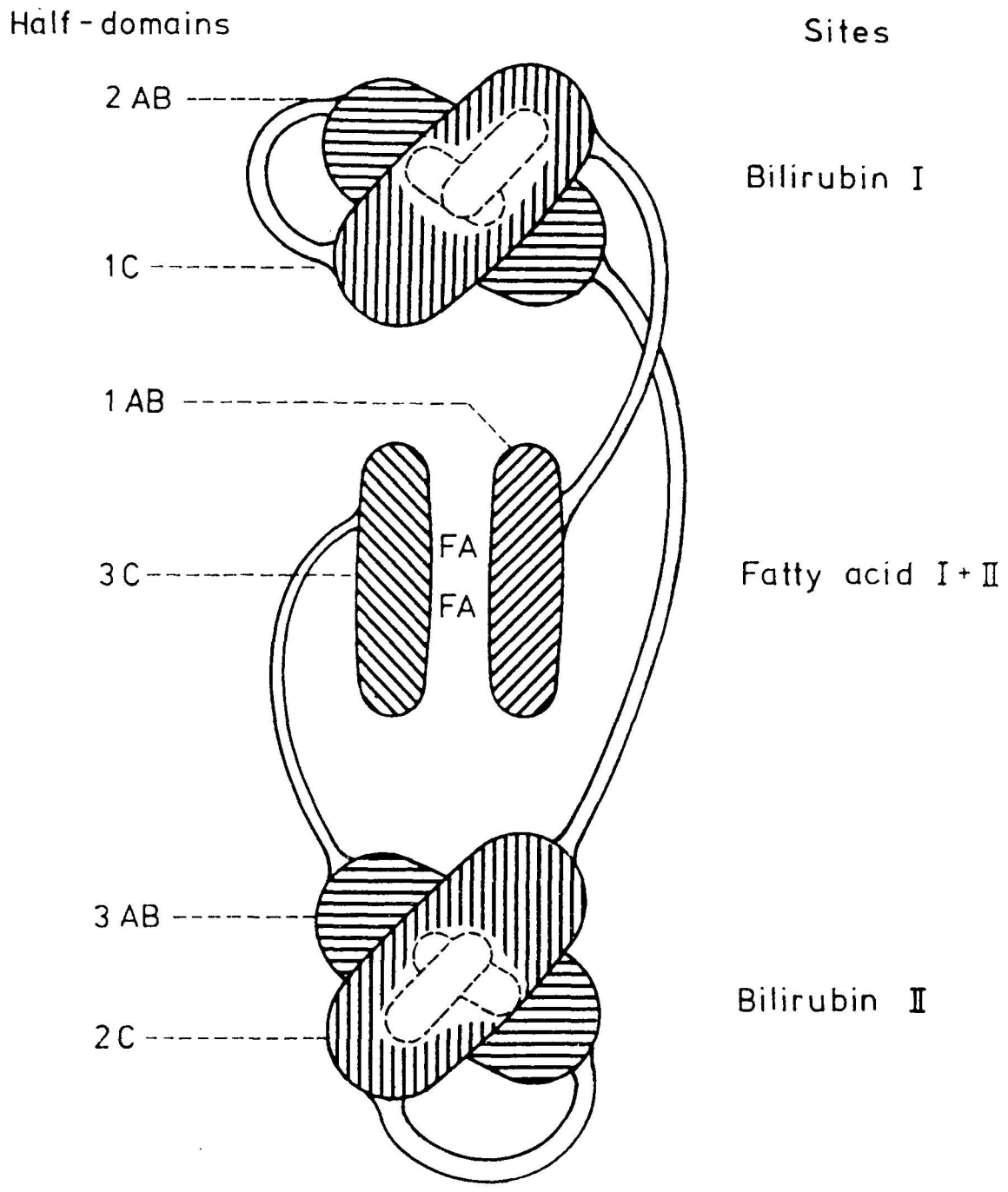


Figure 2. (a) **Stereo view of the heart shaped structure of human serum albumin** with overall topology and secondary structure.
(Adapted from Carter & Ho, 1994)

(b)



(b) **A model of albumin molecule,** showing six half-domains which are combined to form two sites for binding of bilirubin and one for fatty acids (Taken from Brodersen, 1979). According to the new nomenclature, half-domains AB and C now refer to subdomains A and B, respectively (Carter & Ho, 1994).

found to show competitive binding to bilirubin high affinity site on albumin (Brodersen, 1974; Brodersen *et al.*, 1977; Ishimura *et al.*, 1990; Robertson, 1991). These ligands are anionic, contain aromatic structure and are more or less water-soluble. The binding of drugs to plasma proteins may play important role in their pharmacokinetics and pharmacological results. Several authors reported that albumin is the protein contributing most of these interactions (Kragh-Hansen, 1981). The differences in the binding percentages of various ligands have been reported to be due to greater affinity and not the existence of additional sites.

BILIRUBIN-ALBUMIN INTERACTION

Bilirubin exists in serum in either free form (unconjugated bilirubin), or conjugated form (monoglucuronide and diglucuronide bilirubins) or bound to albumin (Kurosaka *et al.*, 1998). The reaction of bilirubin with human serum albumin was first quantitated by equilibrium dialysis (Martin, 1949). Albumin has one strong (primary site) and one weak bilirubin binding site (Figure 2, b) but only the primary binding site is of physiological importance because of the safe delivery of the bound pigment to the liver (Peters, 1992). High affinity bilirubin binding site resides near loop 4 of subdomain II A. Bilirubin at the primary site binds in a highly specific way in the form of dianion. One molecule of bilirubin is bound with high affinity (K_a=1.4×10^8 L/mole) and two additional molecules of the pigment bind with lower affinity (K_a=5.0×10^5 L/mole) (Jacobsen, 1969).

Only the binding at first site is strong enough to prevent bilirubin from entering central nervous system of neonates and causing kernicterus (Brodersen, 1979).

The binding affinity of bilirubin to serum albumin has been reported to be independent of pH from 7.0 to 9.0 (Wennberg & Rasmussen, 1978; Brodersen, 1979). However, albumin does not bind bilirubin above pH 12.0 because of unfolding of albumin below pH 7.0 and due to insolubility of bilirubin at low pH. Further, the affinity of bilirubin binding to albumin for primary binding site has been found to decrease with increasing temperature and ionic strength (Jacobsen, 1977). This suggests the role of electrostatic interactions in the binding. Kinetic studies have revealed that the binding is rapid followed by several relaxation steps of conformational changes as seen in the light absorption spectra of bilirubin upon binding to albumin (Jacobsen & Brodersen, 1983). Binding of bilirubin to albumin has been studied using circular dichroism (CD), nuclear magnetic resonance (NMR) and absorption spectroscopy (Harmatz & Blauer, 1975; Kaplan & Navon, 1981; 1982; 1983; Lightner *et al.*, 1986; 1988) but the conformation of bound bilirubin and nature of binding site on albumin remains poorly understood.

Bilirubin in solution exists in two enantiomeric conformations M and P, which are in dynamic equilibrium as shown in Figure 3 (Manitto & Monti, 1976; Kaplan & Navon, 1983). Albumin binds exclusively to one of the enantiomers depending on the species chosen and induces circular dichroism (CD) in the visible region, in the bound bilirubin. The induced-CD spectra is characterized by the bisignate CD Cotton effects (CDCEs) or monosignate CDCE usually of

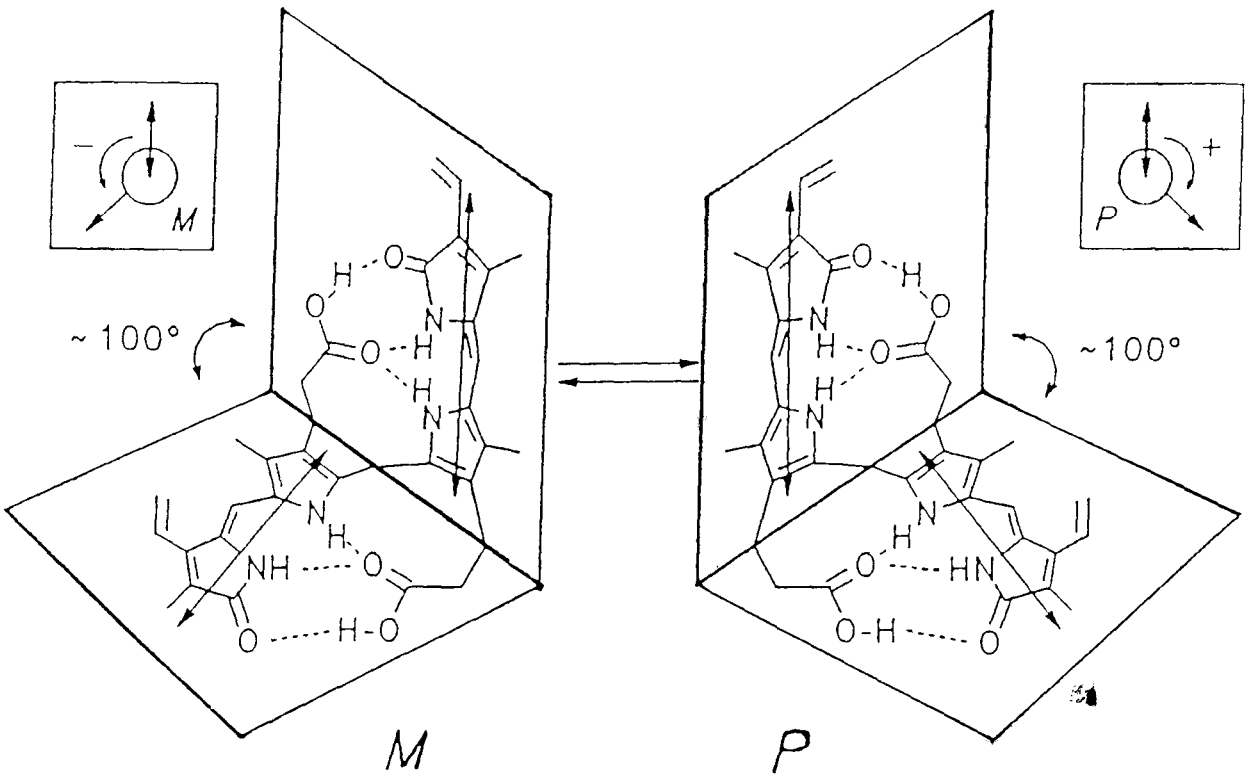


Figure 3. The two ridge-tile conformational enantiomers of bilirubin. The double-headed arrows passing through the dipyrrinone chromophores indicate the orientation of electronic transition dipoles of each dipyrrinone. Insets show the relative helicities (*M*, minus or *P*, plus) of the dipoles (Boiadjiev & Lightner, 1997).

longer wavelength and negative sign (Harmatz & Blauer, 1975). In case of bilirubin complexed with HSA, CD spectra is characterized by the bisignate CDCEs having minima (–) at shorter wavelength and maxima (+) at longer wavelength (Harmatz & Blauer, 1975; Blauer *et al.*, 1977; Lightner *et al.*, 1986; 1988). Bisignate CDCEs thus seem to be characteristic of either a bichromophoric pigment complexed to the protein complex containing two chromophores which are not covalently linked to each other but which presumably bear proximal relationship (Lightner *et al.*, 1986). The observed bisignate CDCEs are due to chromophore-chromophore interaction of the two locally excited states of the twin pyrromethanone chromophores i.e. exciton-splitting (Harada & Nakashami, 1983). The sign order in a bisignate CD spectrum depends on the relative helical orientation of two pyrromethanone electric dipole moments. A right-handed helicity (positive (*P*) chirality) of the transition moments generates a (+) longer wavelength Cotton effect followed by a (–) shorter wavelength Cotton effect as observed in case of bilirubin-HSA complex. On the other hand, left handed helical orientation (negative (*M*) chirality) of the electric dipole moments produces inverted CDCEs (Harada & Nakanishi, 1983) as observed in case of bilirubin-BSA complex (Harmatz & Blauer, 1975). It has been concluded that the interaction of only one of the two carboxyl groups of bilirubin with a single positively charged amino acid residue, such as arginine or lysine, is required to maintain the ridge tile bilirubin conformation observed with bilirubin-HAS complex (Petersen *et al.*, 2000).

BILIRUBIN TOXICITY AND BILIRUBIN ENCEPHALOPATHY

Bilirubin belongs to a class of substances usually termed cholephilic organ anions, because their concentration is 10-1000-fold higher in bile than in blood (Klassen & Watkin, 1984). When bilirubin concentration in plasma exceeds 10 mg/L, hyperbilirubinemia exists and over 350 mg/L, the bilirubin fixation capacity of plasma is overloaded and bilirubin diffuses from the plasma to the tissues, which become yellow, a condition known as jaundice or icterus (Scharschmidt, 1988). The safe upper limit i.e., the threshold for neurotoxicity, of unbound bilirubin is reported to be >20 nmol/L for low birth weight infants (Cashore & Oh, 1982). In hyperbilirubinemic infants, particularly when total bilirubin exceeds 200 μmol/L, unbound bilirubin usually increases to >10 mmol/L (Ihara *et al.*, 1987). Maintenance of unbound bilirubin below 20 nmol/L is important for prevention of icterus-associated neurological deficits. Schmorl (1904) suggested the term 'kernicterus' for yellow staining of basal ganglia in association with neonatal jaundice. This term has been used, since then to describe both the acute, often fatal seizures, opisthotomes and bleeding tendency as well as the neurologic sequelae in survivors, consisting of choreoathetosis, asymmetric spasticity, paresis of upward gaze and neurogenic hearing loss (Byers *et al.*, 1955; Perlstein, 1960; Hyman *et al.*, 1969; Keaster *et al.*, 1969; Fenwick, 1975). But now-a-days, the term 'kernicterus' is replaced by another term 'bilirubin encephalopathy' which includes all conditions where bilirubin is known or thought to be the cause of brain toxicity (Hansen and Bratlid, 1986). Neonatal jaundice is a common transitional phenomenon, which is due to

combination of increased heme catabolism and rate limitations as well as hepatic conjugation and biliary excretion of bilirubin (Hansen, 2000).

Free unconjugated bilirubin is a potentially cytotoxic substance (Metze, 1977). Its damaging effects are seen not only on the cells of nervous system but also in other cell types and tissues e.g. heart, kidney, Langerhan's islets of pancreas, liver, platelets and teeth (Metze, 1977). Bilirubin might be toxic to immune system cells, lymphocytes and granulocytes (Miler *et al.*, 1985). Several enzyme systems are influenced (Karp, 1979; Amit & Boneh, 1993) as inhibitory effects of bilirubin on the binding of cAMP to protein kinase are reported (Morphis *et al.*, 1982). In cultured human fibroblasts, bilirubin combined with phototherapy induces strand breakage in DNA (Rosenstein *et al.*, 1983; Rosenstein & Ducore, 1984). Increased entry of bilirubin into brain is associated with reduced bilirubin binding capacity of albumin due to the presence of displacing agents and opening of blood brain barrier due to hyperosmolality, hypercarbia and asphyxia. Recently, in experimental animals, albumin-bound bilirubin has been observed to enter the brain and further aggravates the risk of bilirubin encephalopathy, which may be facilitated by slight opening of blood brain barrier (Hansen & Bratlid, 1986; Hansen *et al.*, 1987; 1989; Hansen, 1996).

The effects of bilirubin on neurophysiologic processes have only been studied to a limited extent. It has been observed that in the cells, bilirubin is bound to enzyme carrying membranes such as those of mitochondria (Brodersen, 1979). It shows wide spread effects on protein phosphorylation by inhibiting various enzymes, namely cAMP-, cGMP-, Ca^{++}-calmodulin- and Ca^{++}-phospholipid

dependent protein kinases (Hansen *et al.*, 1996). It also inhibits phosphorylation of histones in the brain of 3-4 days old rabbit (Morphis *et al.*, 1982).

Nature of bilirubin involved in bilirubin cell toxicity is still a controversy. It has been suggested that kernicterus and bilirubin toxicity are caused by the bilirubin-albumin complex crossing a disrupted blood-brain barrier and that the level of free bilirubin or nature of bilirubin binding may not be important in the development of bilirubin encephalopathy (Levine *et al.*, 1982). On the other hand, studies on the effects of bilirubin on respiration and viability in cell cultures have shown that toxicity increases when bilirubin is present in the incubate in molar concentrations which exceed that of albumin (Lie & Bratlid, 1970; Bratlid & Rugstad, 1972). Further, if albumin is present in an equimolar or higher ratio with bilirubin, toxicity is blocked (Rasmussen & Wennberg, 1972). Now it is well recognized that free, unconjugated bilirubin is the potential cytotoxic substance and its damaging effect on the cells of the central nervous system in newborns, suffering from a severe form of hyperbilirubinemia (kernicterus) is well known (Odell, 1980; Brodersen & Stern, 1990). It is a commonly accepted fact that the toxicity of bilirubin depends on its passage across the plasma membrane and its association with membrane lipids (Mustafa & King, 1970; Hayward *et al.*, 1986; Noy *et al.*, 1992; Ali & Zakim, 1993; Zucker *et al.*, 1994). During the *in vitro* interaction between bilirubin and cells, bilirubin undergoes degradation due to both adsorption to the cells and action of cell enzymes (Knobloch & Miler, 1987). Recently, Brites *et al.* (1997) have concluded that the first step of erythrocyte bilirubin toxicity is crenation due to

expansion of the outer membrane leaflet by bilirubin monoanion location. This effect is more evident in younger cells and explains the protection against the hypotonic hemolysis. Insertion of bilirubin deeper into the bilayer, facilitated by higher concentration ($\geqslant 1\times10^{-4}$ mol/L) and cell age, produces unstable situation where bilirubin acid aggregation is the main cause for hemolysis and cell destruction. However, dual character of bilirubin in its biological effects has been proposed (McDonagh, 1990). It causes cell damage (Vazquez *et al.*, 1988) on one hand but protects cell against oxidative damage on the other hand (Stocker *et al.*, 1987; Yesilkaya *et al.*, 1998). Bilirubin may also protect mammals from copper poisoning as it acts as an anti-precipitant against copper mediated denaturation of BSA by forming a copper-bilirubin complex (Adhikari *et al.*, 1998).

BINDING OF BILIRUBIN TO ERYTHROCYTES / ERYTHROCYTE MEMBRANES

In the blood of healthy individuals, unconjugated bilirubin is almost entirely bound to albumin. If either the molar concentration of bilirubin exceeds the binding capacity of albumin or various competitive inhibitors for bilirubin binding to albumin like antibiotics, hormones, sulfonamides, salicylates or other drugs are present, the bilirubin may bind additionally to cells of different tissues including erythrocytes (Hayer *et al.*, 1989). Binding of bilirubin to erythrocyte membrane and biological membranes is well known (Thaler & Wennberg, 1977; Bouillerot *et al.*, 1981; Sato & Kashiwamata, 1983; Sato *et al.*, 1987; Gulian *et*

al., 1987; Wennberg, 1988; Hayer *et al.*, 1989; Amit & Boneh, 1993; Ochoa *et al.*, 1993) and has been suggested to be a useful criterion for the risk of bilirubin encephalopathy (Bratlid, 1972a). The uptake of bilirubin by erythrocytes has been found to be dependent on both bilirubin/albumin molar ratio in the serum and total bilirubin concentration (Kaufmann *et al.*, 1967). The bilirubin uptake by erythrocytes increases either with the increase in bilirubin concentration while bilirubin/albumin molar ratio is constant or with the increase in bilirubin/albumin ratio while bilirubin concentration is kept constant (Bratlid, 1972a). However, low concentration of bilirubin (10^{-7}-10^{-5} mol/L) protects the erythrocytes against hypotonic lysis and leads crenation, while higher bilirubin concentration induces hemolysis and leads to membrane disruption (Brites *et al.*, 1997). Bilirubin saturated membranes show membrane rupture due to impairment in lipid-lipid, protein-protein and lipid-protein interactions. Bilirubin tends to aggregate once bound to phospholipids (Gibson & Antonini, 1963) but exists in different states when associated with plasma membrane (Glushko *et al.*, 1982).

Bilirubin binding to erythrocytes takes place within 10 minutes and further increase in incubation time does not result in any increase in erythrocyte-bound bilirubin (Sato & Kashiwamata, 1983). At a given albumin concentration, erythrocytes and albumin bind constant proportions of bilirubin, despite varying bilirubin concentrations (Barnhart & Clarenburg, 1973). However, Wennberg & Rasmussen (1978) have reported that at equilibrium, the cellular content of bilirubin is a function of the concentration of free bilirubin acid and not the total bilirubin concentration or bilirubin/albumin molar ratio.

The effect of pH on the binding of bilirubin to human erythrocytes has been was studied by Bratlid (1972b) and several other workers (Nelson *et al.*, 1974; Sato & Kashiwamata, 1983; Wennberg, 1988). Erythrocyte binding of bilirubin is increased with decrease in pH and hence, jaundiced neonates with low plasma pH have been reported to be at greater risk of developing bilirubin encephalopathy (Kim *et al.*, 1980). In presence of albumin, decrease in pH from 7.4 to 6.8 results in an increase in erythrocyte-bound bilirubin at all the bilirubin/albumin molar ratios, including molar ratios below 1:1 (Bratlid, 1972b). In the absence of albumin, at higher bilirubin concentrations, there is an increase in erythrocyte-bound bilirubin on decreasing pH, although at lower bilirubin concentrations, decrease in pH does not affect the erythrocyte-bound bilirubin (Bratlid, 1972b). Thus, it appears that bilirubin binding to erythrocytes depends on both pH and bilirubin concentration (Bratlid, 1972b). Distribution of unbound unconjugated bilirubin among body fluids and tissues differs for its three ionic species, whose proportions are determined by pH (Ostrow *et al.*, 1994). At pH 7.4, the fully protonated unconjugated bilirubin (di-acid) is the dominant species (83 %) and the monoanion constitutes 16 % but there is less than 1.5 % dianion. As pH increases from 7.0 to 8.0, the proportion of unconjugated di-acid declines and the proportion of monoanion increases steeply; the proportion of dianion, however shows little increase until the pH is above 7.5 and still constitutes only 17 % of unbound unconjugated bilirubin at pH 8.0 (Ostrow *et al.*, 1994). Most of the workers (Zamet & Chunga, 1971; Bratlid, 1972b; Maisels, 1972) are of the view that increased cellular binding of bilirubin is due to increased dissociation

of bilirubin from the secondary binding sites of albumin occurs at low pH without any reduction in the affinity of the primary binding site. Bilirubin is said to bind to various structures of cells apart from the cell membrane as evident from the lesser binding of bilirubin to cell stroma as compared to intact cells (Bratlid, 1972a). Neither storage for one week nor anticoagulant adversely affect the ability of erythrocytes to bind bilirubin.

Although cellular binding of bilirubin is well-known, multisite toxicity of bilirubin towards the metabolism of living cells has not yet been completely elucidated. Also, the nature of bilirubin binding to biological membranes and pathway(s) through which bilirubin enters into the cells is(are) not fully understood. Increased toxicity of bilirubin at a bilirubin/albumin molar ratio higher than 1:1 (Lie & Bratlid, 1970; Bratlid & Rugstad, 1972) suggests that it is free unbound bilirubin and not albumin-bound bilirubin that binds cell membranes. Many workers are of the view that bilirubin diffuses freely through biological membranes as it has been found accumulated in the intracellular compartments such as mitochondria (Odell, 1966; 1969; Wennberg, 1988). Based on the spontaneous leakage of bilirubin from multilamellar liposomes (Hayward *et al.*, 1986), it has been proposed that bilirubin is able to diffuse through cellular membrane (Wennberg, 1988; Schmidt, 1972) though several putative bilirubin transporters have been described (Stremmel & Berk, 1986; Kullak-Ublick *et al.*, 1994). Unconjugated bilirubin has been shown to diffuse through model phosphatidylcholine vesicles as the uncharged diacid (Zucker *et al.*, 1999) (Figure 4, iv). On the other hand, facilitated mode of bilirubin binding

accompanied by a release of phospholipids from the cell membranes favoring the bilirubin penetration and aggregation within the membranes has also been described (Brito *et al.*, 1996). Lamola *et al.* (1979) have shown that bilirubin is not solely bound to membranes but that its greater part is present in the cytosol of erythrocytes. Contrarily, lysed erythrocyte membranes have been shown to bind 14 % more bilirubin than intact ghost (Karp *et al.*, 1985). It seems that unlike other cells, erythrocytes have all the bilirubin bound to the specific binding sites present in the membranes. A binding saturation of 110×10^{-18} moles of bilirubin per erythrocyte and dissociation constant of bilirubin/receptor complexes 170×10^{-6} mol/L (Hayer *et al.*, 1989) has been given. Sato & Kashiwamata (1983) have reported apparent dissociation constant (K_d) as 2.3 μM and maximum binding (B_{max}) value for the saturable binding of bilirubin as 0.93 nmol/mg of membrane protein. Also, binding of bilirubin to membranes result in morphological alteration of erythrocytes (Brites *et al.*, 1997). Loss of high affinity ouabain binding sites on the bilirubin-treated erythrocytes also suggests the presence of membranous bilirubin binding sites (Corchs *et al.*, 1994). The characterization of the individual membrane components responsible for binding of bilirubin and the location of these sites, however, remains to be worked out.

Biomembranes have two important constituents, i.e. lipids and proteins, and bilirubin binding sites are supposed to be made up of either lipids or proteins, or both. Involvement of membrane proteins as bilirubin binding sites has been ruled out in earlier studies (Sato & Kashiwamata, 1983; Chen *et al.*, 1992) based on increase in bilirubin binding capacity after tryptic digestion and heat treatment of

(i)

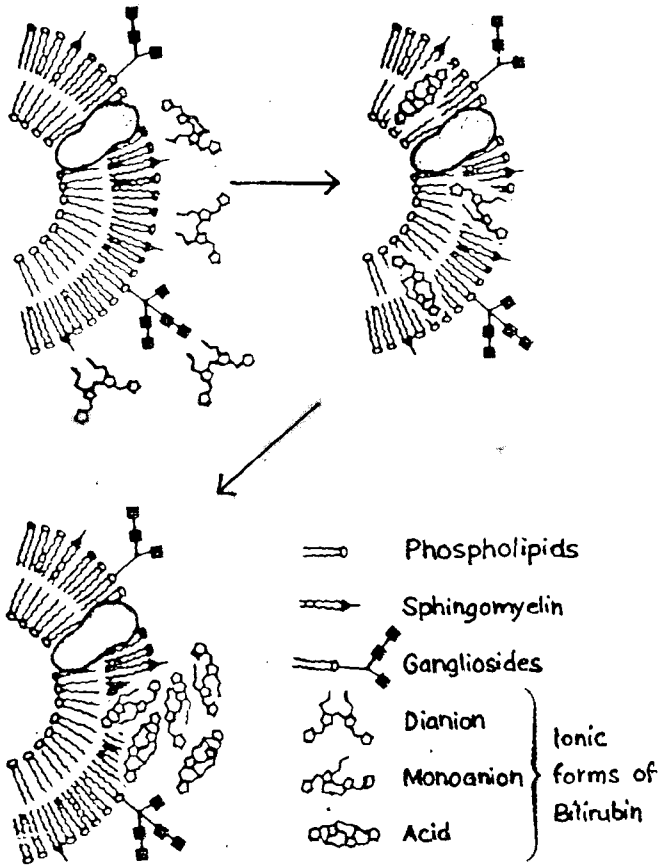
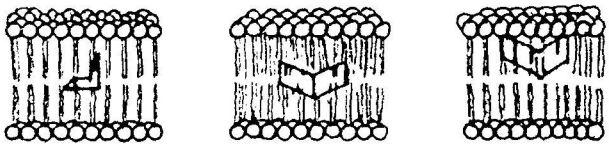


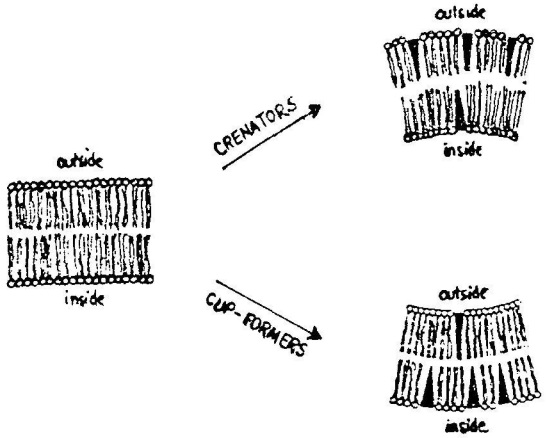
Figure 4. Models proposed for the orientation/localization of bilirubin within the bilayer

(i) Model proposed for the interaction of bilirubin with synaptosomal membrane (as given by Vazquez *et al.*, 1988)

(ii)



(iii)



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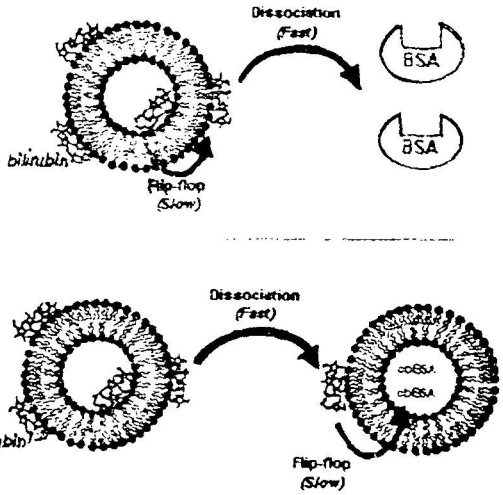


(ii) Internally hydrogen bonded structure of bilirubin IX α can have only three possible orientations in a lipid bilayer (as given by Zakim & Wong, 1990).

(iii) Schematic representation of the proposed binding of amphipathic compounds that are crenators or cup-formers to the phospholipid regions of the erythrocyte membrane (Taken from Sheetz & Singer, 1974).

(iv) Schematic illustration of experimental systems utilized for studying bilirubin transmembrane diffusion depicting that unconjugated bilirubin exhibits spontaneous diffusion through lipid bilayers and native hepatocyte membranes (Zucker *et al.*, 1999).

membranes and failure in the affinity labeling of any membrane proteins (Sato & Kashiwamata, 1983). It has been suggested that proteins may function as effective barrier to the binding of bilirubin. Contrarily, Leonard *et al.* (1989) have suggested that membrane proteins may exert some type of non-specific effect on the properties of the apolar phase of the bilayer, enhancing the capacity of the bilayer to sequester more bilirubin as compared to protein-free bilayer. However, it is widely accepted that membrane lipids and not proteins are the bilirubin binding sites. Vazquez *et al.* (1988) working on synaptosomal plasma membranes reported that bilirubin binding sites are mainly located on the outer layer of bilayer membrane. However, Karp *et al.* (1985) have suggested that bilirubin binds to both outer and inner surface of the membrane. This has also been supported by Sato *et al.* (1987) who showed no difference in bilirubin binding capacity of inside-out and right-side out sealed membranes.

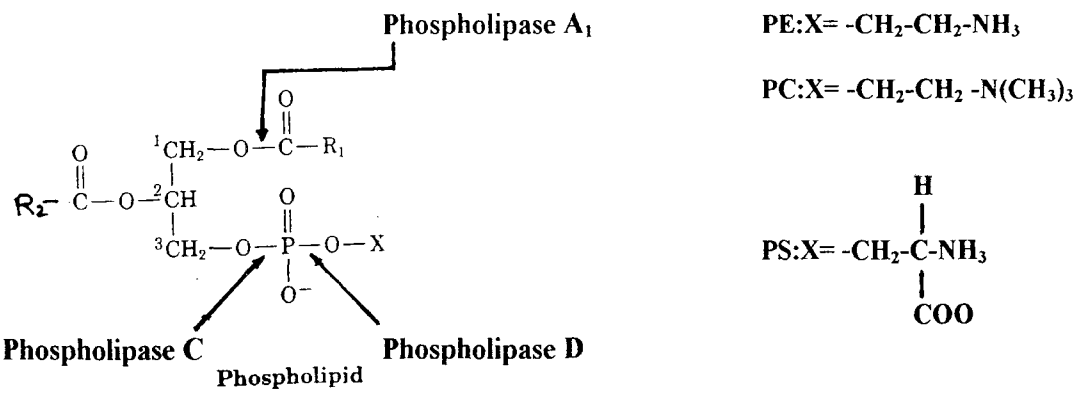
The mechanism of interaction of bilirubin with the lipid bilayer of erythrocyte membranes is still not clear. While Sheetz & Singer (1974) proposed that anionic or impermeable amphipathic compounds intercalate with lipids in the exterior half of the bilayer (Figure 4, iii) causing expansion of that bilayer relative to cytoplasmic half and induce crenation of cells, Nagaoka & Cowger (1978) have suggested that bilirubin is bound to the lipid bilayer through strong ionic interactions between a cationic head group of the lipid and anionic bilirubin. Binding constant has been reported to be highest for sphingomyelin as 3.7×10^{6} M^{-1} and diphosphatidylcholine with 5 % cholesterol as 2.6×10^{6} M^{-1}. Cestaro *et al.* (1983) have suggested that the dianion form of bilirubin binds weakly to polar

head groups of phospholipids whereas acid bilirubin binds strongly and is hydrophobically inserted into the lipophilic region of the bilayer. This hydrophibically bound bilirubin can perturb the chemico-physical properties such as fluidity, phase transition (Figure 5a) etc. of the membrane (Cestaro *et al.*, 1983).

Binding of bilirubin to synaptosomal plasma membrane vesicles isolated from rat brain is suggested to occur in three steps (Vazquez *et al.*, 1988) (Figure 4, i):

(i) a rapid formation of an electrostatic complex between bilirubin and lipid polar head groups on the membrane surface, especially those from gangliosides and sphingomyelin; (ii) a slow inclusion of the pigment into hydrophobic core of the membrane and (iii) a membrane-induced aggregation of bilirubin acid.

The protruding ends of the lipids are supposed to be involved in the initial interaction with bilirubin (Vazquez *et al.*, 1988) as treatment of membrane with phospholipases C and D resultes in a decrease and an increase in bilirubin association constant, respectively. However Sato *et al.* (1987) have found that phospholipase D treatment (which removes polar head groups of PC, PE and PS to form phosphatidic acid as shown below)



Action of phospholipases

does not change the bilirubin binding capacity of membranes whereas phospholipase C treatment enhances the bilirubin binding greatly. Therefore, the saturable bilirubin binding sites are probably located at positions on membranes that are not composed of polar head groups of phospholipids rather, the negatively charged phosphoric acid moiety of phospholipids prevents a large amount of bilirubin binding to the membranes. Leonard *et al.* (1989) are of the opinion that bilirubin interacts with neither the polar region nor the apolar regions of membranes but is localized within the membrane bilayer due to voids in the packing of bilayer. Zakim & Wong (1990) have reported that bilirubin is intercalated tightly into the polymethylene chain region in the bilayer (Figure 4, ii) whereas other views present the involvement of hydrogen bonding in bilirubin membrane interaction (Noy & Xu, 1990; Zucker *et al.*, 1992). Further suggestions have been made that a major portion of bilirubin molecule is not localized within the hydrophobic domain of the bilayer but rather associated at the membrane surface and the dissociation of the membrane-bound bilirubin depends upon the phospholipid composition of the membranes and the membrane size (Zucker *et al.*, 1992; 1994). Recently, Brites *et al.* (1997) have suggested that bilirubin monovalent anion is the species first involved in the interaction with the erythrocyte membrane, the single ionized carboxyl group located in the aqueous medium, while the hydrophobic portion is buried in the bilayer, resembling a detergent.

The influence of physico-chemical properties of the membranes such as overall charge on membrane surface, membrane fluidity (Figure 5a), pH and

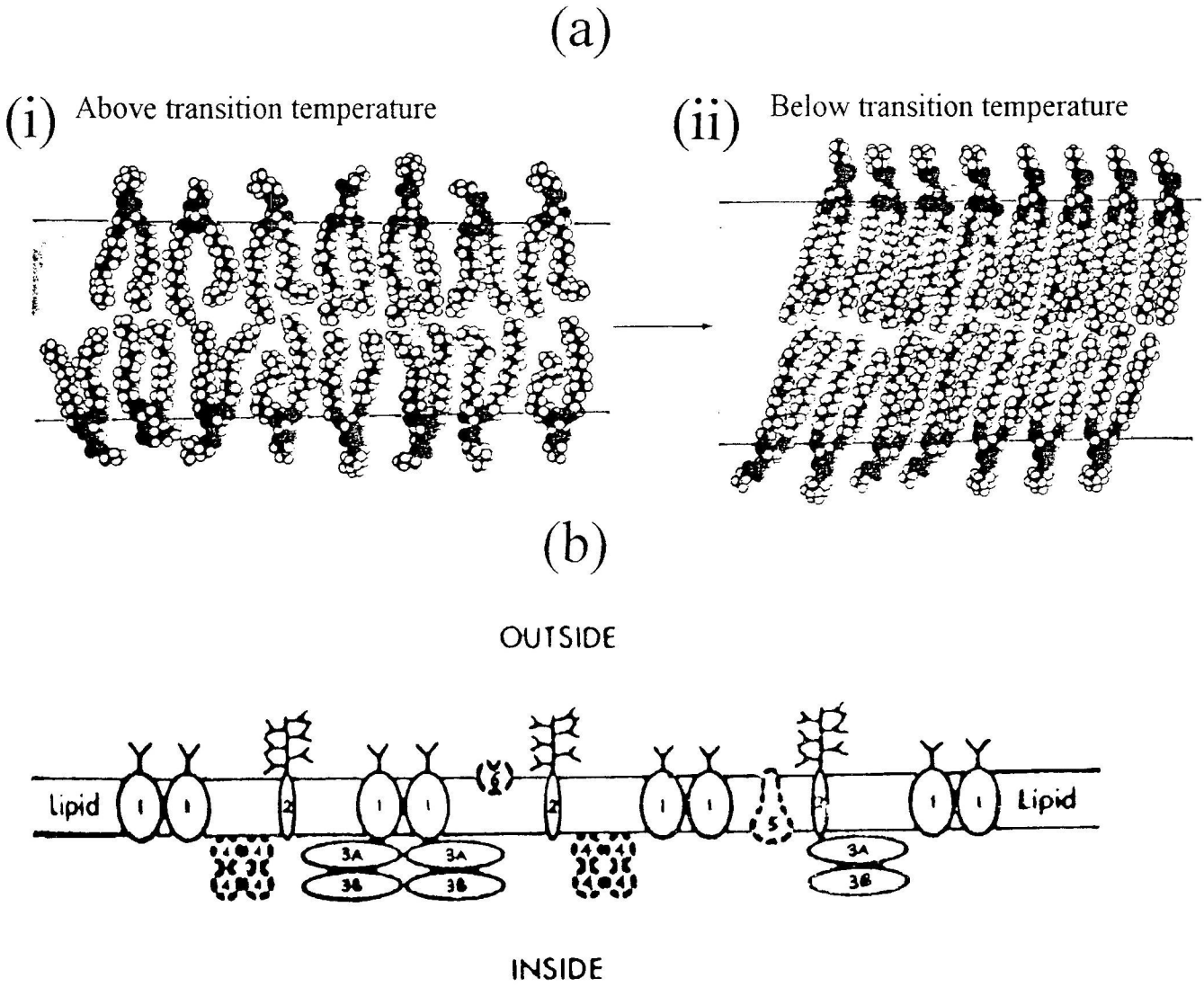


Figure 5. (a) Phase transition in a lipid bilayer.

(i) Above the transition temperature, both the lipid molecules as a whole and their nonpolar tails are highly mobile in the plane of the bilayer.
(ii) Below the transition temperature, the lipid molecules form a much more orderly array to yield a gel-like solid. (Adapted from Voet, Voet & Pratt, 1998).

(b) A model of the erythrocyte membrane.

1. 100 000 mol. wt protein 2. Sialoglycoprotein 3. Spectrin 4.Glyceraldehyde-3-phosphate dehydrogenase 5. (Na^+-K^+)-ATPase 6.Acetylcholinesterase.
(Adapted from Juliano, 1973).

temperature on the binding of bilirubin to the membranes are of great concern. Vazquez *et al.* (1988) have suggested that the interaction of bilirubin with membrane is modulated by membrane fluidity such that lesser the fluidity, lesser the binding of bilirubin to membrane. Karp *et al.* (1985) are of the view that change in shape and size greatly affects the binding of bilirubin as seen by the expansion of erythrocyte membranes by a drug, chlorpromazine, which enhances bilirubin uptake of membranes by 45 %. Saturable binding of bilirubin to erythrocyte membranes has a pH optimum at around 7.1 (Sato & Kashiwamata, 1983). A U-shaped thermal dependency of the total and saturable binding of bilirubin to erythrocyte membranes is reported with a minimum value near 37°C and gradual increase below and above this temperature (Sato & Kashiwamata, 1983). Contrary to this, Leonard *et al.* (1989) have reported no such thermal dependency of the binding of bilirubin to biological membranes over a temperature range of 10-40°C. Addition of calcium chloride to the assay medium increases the binding of bilirubin to membrane (Sato *et al.*, 1987; Vazquez *et al.*, 1988). In view of the binding of calcium to the sialic acid residues of membrane (Moore *et al.*, 1984), Vazquez *et al.* (1988) have suggested that negative charges present on the sialic acid residues exert an inhibitory effect on the binding of bilirubin to membranes. Addition of calcium ions shields the negative charges of sialic acid residues, thereby increasing bilirubin binding. On the other hand, no significant change in bilirubin binding after neuraminadase treatment of membranes suggest that negative charges of sialic acid residues play little role in the binding of bilirubin to membrane. Recently, Brito *et al.* (2000) have shown

that increased lipid fluidity and high bilirubin concentration promote membrane bilirubin translocation and toxicity. At low concentrations (nM), bilirubin prefers water-membrane interface, however, when concentration is increased to μM range, it is situated in the inner core as well. Whether or not it diffuses across membrane bilayer needs more precise information. The temperature-dependent differences suggest that unconjugated bilirubin is differently located, or in a different physical state. However, albumin is not able to displace the bilirubin located deeply or aggregated within the membrane because of the conformational changes occuring by temperature in albumin (Brodersen, 1979) which increases the co-crystallization of unconjugated bilirubin and HSA. It has also been reported that antioxidant defence property of erythrocyte membrane is altered in jaundiced neonates (Mazumder *et al.*, 1995). Studies in newborn humans have demonstrated alteration in the lipid, phospholipid and cholesterol content when compared with age-matched control. Membrane-bound (Na^+-K^+)ATP-ase activity is found to be significantly increased in jaundiced neonates. Alteration in membrane permeability characteristics in jaundiced neonates causes severe *microenvironmental changes in erythrocyte profile (Mazumder et al., 2000).*

ERYTHROCYTE MEMBRANE STRUCTURE

It is generally agreed that the fluid mosaic model of membrane structure (Singer, 1974) provides a satisfactory general description of the molecular organization of the proteins and lipids of most functional membranes (Figure 6a). In this model, the integral proteins are amphipathic molecules with their hydrophobic ends

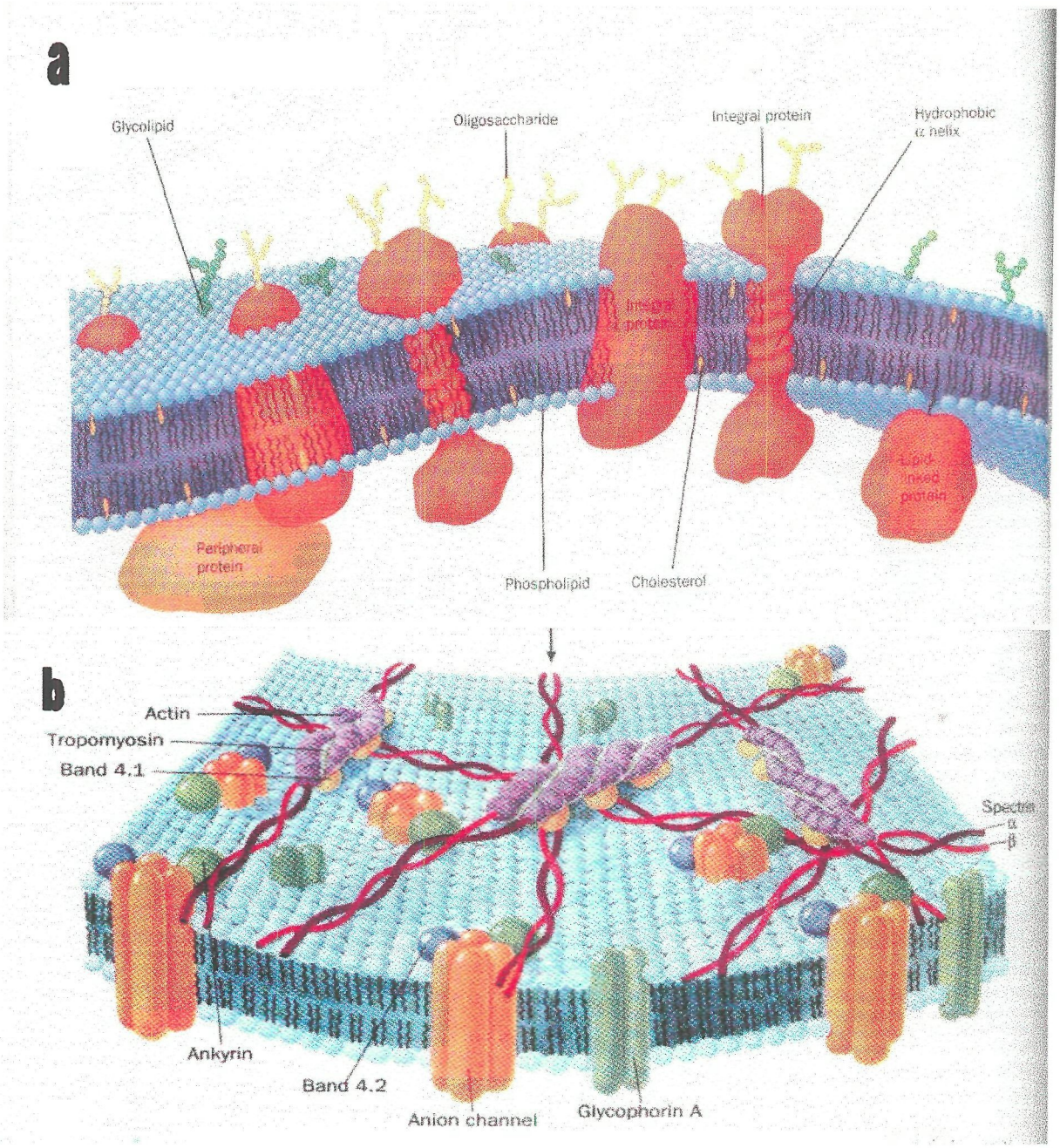
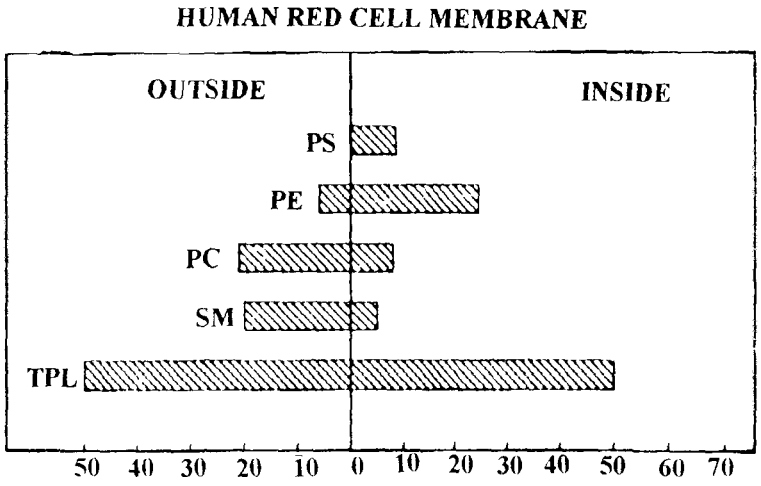


Figure 6. **(a) Diagram of plasma membrane**. Integral proteins are embedded in a bilayer composed of phospholipids and cholesterol. The carbohydrate components of glycoproteins and glycolipids occur only on the external face of the membrane.

(b) A model of erythrocyte membrane skeleton, showing junctional complex containing actin, tropomyosin and band 4.1 protein as well as other proteins.

(Adapted from Voet, Voet & Pratt, 1998).

embedded in the matrix of the membrane and their hydrophilic ends protruding from it. The matrix of the membrane is a bilayer of phospholipids, at least some part of which is fluid under physiological conditions. There is increasing evidence that both proteins and the phospholipids of membranes are asymmetrically distributed in the two halves of bilayer.

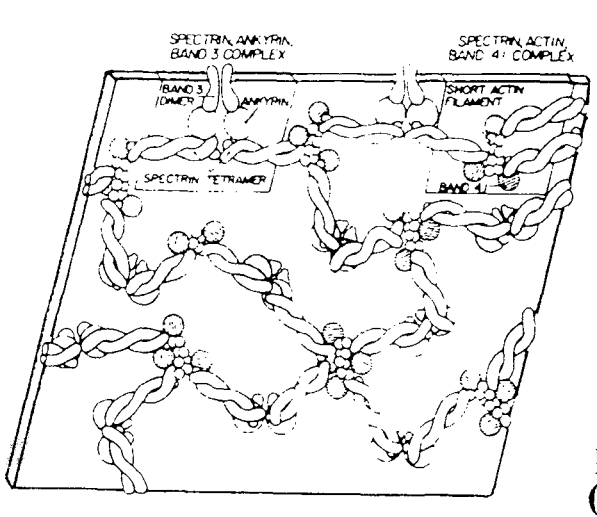


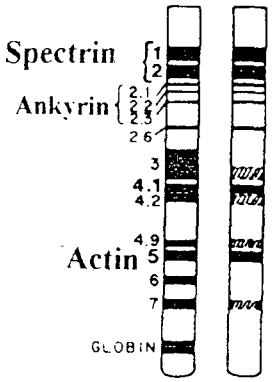
Asymmetrical distribution of phospholipids in human red blood cells expressed as mole percent (TPL, total phospholipid;PC, phosphatidyl choline;SM, sphingomyelin, PE, phosphatidyl ethanolamine; PS, phosphatidylserine and PI, phosphatidylinositol)

(Adapted from Voet, Voet & Pratt, 1998)

This evidence is most substantial for the erythrocyte membrane with respect to its proteins (Bretscher, 1973) and phospholipids (Zwaal *et al.*, 1973). Phosphatidylcholine (PC) and sphingomyelin (Sph)(choline-containing phospholipids) appear to be concentrated in the exterior half of the bilayer, and phosphatidylethanolamine (PE) and phosphatidylserine (PS)(amino phospholipids) in the cytoplasmic half (Bretscher, 1972; Op den Kamp, 1979). This typical transbilayer phospholipid asymmetry is disturbed in erythrocyte containing defective membrane skeleton (Williamson *et al.*, 1982; Kumar & Gupta, 1983). Heating increases protein-protein association within the skeleton

but membrane bilayer-skeleton interactions are not enhanced (Kumar *et al.*, 1990). To survive in the circulation erythrocytes must be both durable and flexible, durable enough to withstand the turbulent cardiac passage and flexible enough to negotiate the narrow portals of the spleen. These dual demands are the responsibility of a sub membranous protein network functioning as membrane skeleton. Human erythrocyte membrane is composed of two structural units, viz. membrane skeleton (or cytoskeleton) and membrane bilayer; of which the cytoskeleton is formed from three major (spectrin, actin and band 4.1) and





SDS polyacrylamide gel pattern of red cell membrane (ghosts) and membrane skeletons

Postulated organization of the major skeletal proteins

A. Polypeptides	Band	Mol. Wt.
1	(I+II)	240,000
2	*Spectrin	215,000
3	III	88,000
4.1	IV	78,000
4.2		72,000
*Region 4.5		*45,000
5	V *(Actin)	43,000
6	VI *(Glyceraldehyde-3-phosphate dehydrogenase)	35,000
7		29,000
B.Glycoproteins		
PAS-1		55,000
PAS-2		

(Adapted from Steck, 1974) * Taken from Jones & Nickson, 1981

The major erythrocyte membrane polypeptides and glycoproteins

several minor peripheral membrane proteins and is associated with the cytoplasmic face of the membrane bilayer through protein-protein and protein-phospholipid interactions (Bennett, 1985). About 60 % of the membrane-protein mass comprises of membrane-skeleton including spectrin (bands 1 and 2), actin (band 5), ankyrin (bands 2.1, 2.2, 2.3 and 2.6) and band 4.1; and a portion of proteins designated bands 3, 4.2, 4.9 and 7.

The membrane bilayer-skeleton association controls not only the membrane stability and deformability but also the lateral mobility of the integral membrane proteins (Sheetz *et al.*, 1984). Besides, this association is considered to be a major factor in maintaining the asymmetric phospholipid distribution across the erythrocyte membrane bilayer (Haest, 1982). Membrane skeleton is shown to stabilize amino phospholipid distribution in the inner monolayer (Middelkoop *et al.*, 1989) due to its interaction with phosphatidylserine (Shiffer *et al.*, 1988; Rybicki *et al.*, 1988). Also, membrane protein self-association is found to depend on the lipid composition of the membrane (Rothberg *et al.*, 1990). Further, proteins like band 3 and $Na^{+}K^{+}$-ATPase are reported to be strongly associated with phospholipids (Rodgers & Glaser, 1993). Band 3 is the most abundant integral membrane protein and can perturb approximately 700 phospholipid molecules in a bilayer (Chicken & Sharon, 1984). The polypeptides of the erythrocyte membrane can be classified into two major groups (Figure 6, b):

(i) extrinsic (peripheral) membrane proteins which are isolated from membrane by manipulation of ionic strength or pH (the peptides corresponding to bands 1,2,4,5 and 6). Moreover, two kinds of this membrane proteins are: (a) tightly

bound- not eluted with physiologic saline *in vitro* such as ankyrin (band 2.1) which also binds to spectrin and links the submembrane skeleton to the membrane and band 4.2 and (b) loosely bound- readily eluted with physiologic saline *in vitro* such as glycolytic enzymes (phosphofructokinase, aldolase and glyceraldehyde-3-phosphate-dehydrogenase).

(ii) intrinsic (integral) membrane proteins: requires detergents or strong chaotropic agents for their separation as they are more intimately bound (bands 3, 7 and PAS-1, 2, 3and 4).

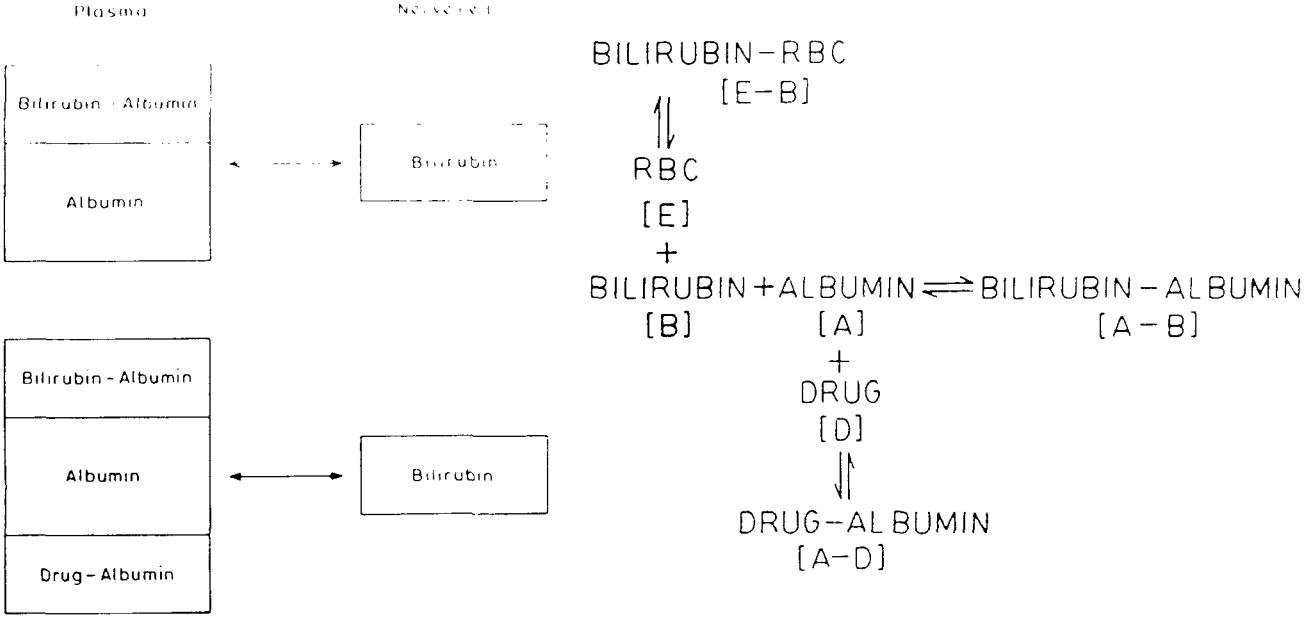
Circular dichroism (CD) studies of membranes revealed that the portions of erythrocyte membrane proteins that are embedded in the lipid bilayer contain a very high (86-94 %) content of α-helix (Oikawa *et al.*, 1985). Recently, CD has been widely used for studying the conformation and conformational change of proteins and polypeptides in solution. The use of phospholipases has turned out to be a valuable tool for investigation of the function of membrane phospholipids and their distribution in the membrane structure (Op den Kamp, 1979), mostly human erythrocyte membrane bilayer (Witt & Gercken, 1986). Membrane permeation of small molecules is the basis of passive drug and metabolite uptake. In order to traverse a lipid bilayer membrane, a solute must enter through the polar head group region diffuse within the hydrocarbon region and then leave

through the polar head group region on the other side (Bassolino-Klimas *et al.*, 1993). The development of new drug delivery systems has taken advantage of the

physiological nature of the erythrocytes to use these cells as carriers (Tonetti and Flora, 1993).

DRUG-INDUCED BILIRUBIN DISPLACEMENT FROM ALBUMIN AND ITS EFFECT ON ERYTHROCYTES

The transfer of bilirubin from plasma to cells depends on the ratio of unconjugated plasma bilirubin concentration to the concentration of reserve albumin and is increased when part of the albumin is either occupied by competitive binding of a drug or unable to bind bilirubin such as at low pH values.



Proposed mechanism of drug-induced bilirubin transfer from plasma to nerve cell (Brodersen et al., 1983) and partition of unconjugated bilirubin in the blood

Silverman *et al.* (1956) have reported increased frequency of kernicterus after administration of combination of sufisoxazole and penicillin. Odell (1959) has explained this as a result of displacement of bilirubin from binding to serum

albumin. Lee & Cowger (1974) have believed that displacement of bilirubin bound to the secondary sites on albumin is more likely than displacement from the primary high affinity site. Some drugs are tightly bound to serum albumin in the blood and some are bound to a lesser degree. The concentration of the drug must be larger than 15 μM to cause significant bilirubin binding competition and based on the chemical nature, most displacing drugs are anionic or without a net electric charge but none of the displacers are lipophilic (Robertson *et al.*, 1991). A displacement constant (K_D) has been calculated by Robertson and his associates (Robertson *et al.*, 1991) which represents the competitive effect of the drug with bilirubin for albumin binding. A maximal displacement factor, δ is calculated from K_D value using the following equation:

$$\delta = K_D\, d + 1$$

where 'd' is the concentration of free drug in patients' plasma. If d=1 or K_D=0 means "no displacement" and if d=1.2, it means that 20 % increase of free bilirubin concentration. Brodersen (1978) suggested that d=1.2 could be taken as an upper limit for permissible degrees of bilirubin displacement. The maximal displacement factor (δ) for ceftriaxone and sodium salicylate has been given as 3.00 and 1.5 and their K_D values as 1.5×10^4 L/mol and 0.92×10^4 L/mol, respectively (Brodersen, 1978). Binding of bilirubin to erythrocytes in a mixture of bilirubin, albumin and drug has been used as a method for studying the bilirubin displacing action of drugs (Bratlid, 1973). The three steps involved in the displacement mechanism are the dissociation of bilirubin from bilirubin-albumin complex; transfer of bilirubin from blood plasma to nerve cells and

failure of intracellular bilirubin disposition by oxidases. Drugs could influence all the three steps and the whole mechanism of drug-induced kernicterus may thus, seem to be quite complex. According to Gulian *et al.* (1986), three forms of bilirubin are present in blood vessels of hyperbilirubinemic neonates: (1) major part of bilirubin is bound to serum albumin (2) linked to erythrocyte lipid membrane and (3) unbound bilirubin, which is the free soluble form. All the three forms are in equilibrium with each other. Displacement of bilirubin to brain requires the opening of blood-brain barrier.

The uptake of bilirubin by human erythrocyte ghosts occurs in the presence of several drugs used clinically in neonates and distinction has been made between effects caused by drug-albumin-bilirubin interactions, ghost lysis, and direct action of the drug on the erythrocyte membrane. A drug may displace bilirubin from albumin (Brodersen, 1978), cause nonhemolytic membrane changes, such as membrane expansion (Kwant & Seeman, 1969) or endocytosis (Schrier & Junga, 1981) or hemolyze the erythrocyte (Seeman, 1966). Drug binding to erythrocytes is of significance in understanding their distribution and other pharmacokinetic parameters. Bilipid membrane of erythrocytes is perforated with pores of various diameter which act as aqueous channels. Drugs can enter cells either by passing through bilipid membrane or through aqueous channels. A positive correlation between partition coefficient and binding suggests that salicylates enter cells through bilipid membrane and not through aqueous channels (Rao & Kumar, 1985). Also, study performed on blood samples from icteric neonates, showed that the addition of ceftriaxone produced an increase in

free bilirubin and erythrocyte-bound bilirubin and a decrease in unconjugated bilirubin (Gulian *et al.*, 1987).

A brief review of literature suggests that these studies are limited in the data on the behavior of bilirubin binding to membranes at various B/As (which are known to exist under physiological and jaundiced conditions) and at different pH and temperatures. In this study, the effect of pH and temperature on the binding of bilirubin to human erythrocyte membranes at different B/As has been reinvestigated since most of the earlier studies on the binding of bilirubin to membranes were carried out either in albumin-free medium (Cestaro *et al.*, 1983) or at a constant B/A i.e., 2.0 (Sato & Kashiwamata, 1983; Sato *et al.*, 1987; Vazquez *et al.*, 1988). From the above review on the bilirubin binding to erythrocyte membranes/biological membranes, it also appears that more studies are required to confirm the localization of bilirubin in the erythrocyte membranes in terms of surface binding, internal binding, hydrophobic binding etc. Therefore, we have also studied the interaction of bilirubin with different types of erythrocyte membrane vesicles such as unsealed, heterogeneous, sealed and inside-out vesicles of two mammalian species namely, human and goat. Further, the effect of phospholipases C and D, trypsin, neuraminidase and Ca^{2+} on the mode of binding of bilirubin to erythrocyte membranes has been studied in detail. Attempts have also been made towards identifying the role of membrane proteins in bilirubin binding phenomenon after the removal of membrane proteins with specific reagents and studying the interaction of these protein-depleted membranes with bilirubin. Visible CD spectroscopy has also been employed

towards the study of interaction of bilirubin with sealed and HSA-entrapped sealed membranes.

Materials & Methods

MATERIALS AND METHODS

[A] MATERIALS

1. Proteins

Bovine serum albumin, fraction V (lot 100F0249), neuraminidase, type V from *Clostrium perfringens* (lot 31H82302), phenylmethylsulfonylfluoride, PMSF (lot 67H1645), phospholipase C, type XIV from *Clostridium welchii* (lot 55H6334), phospholipase D, type I from cabbage (lot 57H0373) and trypsin, TPCK-treated from bovine pancreas (lot 117H7261) were purchased from Sigma Chemical Company, MO, USA. Human and goat serum albumins were isolated from their respective plasma by ammonium sulfate fractionation method (Tayyab & Qasim, 1990).

2. Reagents used in protein estimation

Analytical grade samples of copper sulfate, hydrochloric acid, lithium sulfate, orthophosphoric acid, sodium carbonate, sodium molybdate, sodium potassium tartarate and sodium tungstate were obtained from Qualigens Fine Chemicals, Mumbai, India. Liquid bromine was procured from Sigma Chemical Company, MO, U.S.A. Folin-phenol reagent was prepared according to the standard procedure (Folin & Ciocalteu, 1927). Copper reagent was prepared fresh by mixing 1 volume of 4 % (w/v) sodium potassium tartarate to 100 volumes of 4 % (w/v) sodium carbonate and finally adding 1 volume of 2 % (w/v) copper sulfate.

3. Reagents used in membrane modification

Dimethylmaleic anhydride (lot 77H2521), 3,5-diiodosalicylic acid, lithium salt (lot 48H0714), 2-mercaptoethanol (lot 53H0741) and 1,10-phenanthroline (lot 77H0316) were purchased from Sigma Chemical Company, MO, USA. Copper sulfate (AR), disodium salt (AR) and ethylenediaminetetra acetic acid (AR) were supplied by Qualigens Fine Chemicals, Mumbai, India. Sodium iodide (LR) was the product of Loba Chemie, Mumbai, India.

4. Reagents used in bilirubin estimation

Bilirubin (GR), caffeine anhydrous (LR), sulfanilic acid (LR), sodium benzoate (GR) and sodium nitrite (AR) were the products of S.D. Fine Chemicals, Boisar, India. Sodium acetate (AR) and sodium hydroxide (GPR) were obtained from Qualigens Fine Chemicals, Mumbai, India.

5. Reagents used in gel electrophoresis

Acrylamide (3x crystallized, LR) and N, N'-methylenebisacrylamide (3x crystallized, AR) were purchased from Sisco Research Laboratories, Mumbai, India. Coomassie brilliant blue, R 250 (type B-7920), glycine, LR (lot 113H12551) and trizma base (tris [hydroxymethyl] aminomethane, AR) (lot 14H5717) were obtained from Sigma Chemical Company, MO, U.S.A. Analytical grade samples of ammonium persulfate, acetic acid, glycerol, methanol and sucrose were procured from Qualigens Fine Chemicals, Mumbai,

India. N, N, N', N'-tetramethylethylenediamine (TEMED) was the product of Fluka, AG, Switzerland. Bromophenol blue was obtained from B.D.H., Poole, England. Carbocyanine dye, Stains-all (SA-1) was the product of Eastman Fine Chemicals, New York, U.S.A (No. 2718) and formamide, extrapure AR was obtained from Sisco Reasearch Laboratories, Mumbai, India.

6. Reagents used in inorganic phosphorus estimation

(a) **Fiske & Subbarow (1925) method:** L-Ascorbic acid (AR) was obtained from Sisco Reasearch Laboratories, Mumbai, India. Ammonium molybdate (LR), perchloric acid (LR) and sodium dihydrogen orthophosphate (AR) were the products of Qualigens Fine Chemicals, Mumbai, India.

(b) **Ames & Dubin (1960) method:** Magnesium nitrate (LR) and sulfuric acid (LR) were the products of Qualigens Fine Chemicals, Mumbai, India. Ethanol absolute (GR) was obtained from E.Merck, Darmstadt, Germany.

7. Reagents used in thin-layer chromatography (TLC)

Acetone, extrapure (AR), benzene, extrapure (AR), chloroform (AR) and isopropyl alcohol, extrapure (AR) were purchased from Sisco Research Laboratories, Mumbai, India. Liquor ammonia solution (AR) and iodine (LR) were the products of Qualigens Fine Chemicals, Mumbai, India.

8. Reagents used in sialic acid estimation

Thiobarbituric acid (GPR) was purchased from B.D.H., Poole, England. N-acetyl neuraminic acid, type IV-S (lot 33H78141) was obtained from Sigma Chemical Company, MO, U.S.A. Ammonium periodate (AR) and sodium sulfate (LR) were the products of Qualigens Fine Chemicals, Mumbai, India. Sodium arsenite (GR) was purchased from Loba Chemie, Mumbai, India.

9. Reagents used in carbohydrate estimation

Orcinol (extrapure) was supplied by Sisco Research Laboratories, Mumbai, India. Dextrose (LR) and sucrose (LR) were obtained from Qualigens Fine Chemicals, Mumbai, India.

10. Reagents used in choline estimation

Ammonium reineckate and choline chloride, extrapure were purchased from Loba Chemie, Mumbai, India. Phenophthalein was obtained from Qualigens Fine Chemicals, Mumbai, India. n-Propanol was the product of Sisco Research Laboratories, Mumbai, India.

11. Drugs

Ceftriaxone (lot 82H0034) was obtained from Sigma Chemical Company, MO, U.S.A. Sodium salicylate (AR) was purchased from Central Drug House, New Delhi, India.

12. Miscellaneous

Ammonium sulfate (LR), calcium chloride (LR), di-sodium hydrogen phosphate (LR), potassium hydrogen phthalate (LR), sodium chloride (AR), sodium dodecyl sulfate (LR), sodium dihydrogen phosphate (LR) and sodium tetraborate (LR) were obtained from Qualigens Fine Chemicals, Mumbai, India. Magnesium sulfate was the product of E.Merck, Mumbai, India and tri-sodium citrate (LR) was procured from B.D.H., Mumbai, India.

Fresh human blood (B-Rh+ve) was collected from donors while outdated human blood in 1.32% sodium citrate and 1.47% dextrose was supplied by the Blood Bank of Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh. Goat blood was collected from the local slaughterhouse in 1.32% sodium citrate.

Silica impregnated aluminium sheets (20×20 cm, silica gel 60F254) were from E.Merck, Darmstadt, Germany. Dialysis membrane tubing of 1inch width was obtained from Sigma Chemical Company, MO, U.S.A. Millipore filters (pore size 0.45 μm) were purchased from Millipore (India) Pvt. Ltd., Bangalore, India. Whatman filter papers (No. 1) were the product of Whatman International Ltd., Maidstone, England. Parafilm 'M' was obtained from American Can Company, CT, U.S.A. pH indicator papers were supplied by Qualigens Fine Chemicals, Mumbai, India.

All glass-distilled water was used throughout these studies. All the experiments were performed at room temperature (~25°C) unless otherwise stated.

AR= Analytical Reagent GR= Guaranteed Reagent

LR= Laboratory Reagent GPR=General Purpose Reagent

[B] METHODS

1. pH measurements

pH measurements were carried out on an Elico digital pH meter, model LI 610 using a combined electrode, type CL-51. The least count of the pH meter was 0.01pH unit. The pH meter was routinely calibrated at room temperature with either 0.05 M potassium hydrogen phthalate buffer, pH 4.0 in the acidic range or 0.01 M sodium tetraborate buffer, pH 9.2 in the alkaline range.

2. Optical measurements

Cecil double beam spectrophotometer, model CE594 was used for light absorption measurements in the ultraviolet (UV) as well as visible regions using quartz and glass cuvettes, respectively. Absorption measurements in the visible range were also made on AIMIL Photochem-8 colorimeter, using glass cuvettes of 1cm pathlength.

3. Determination of protein concentration

Protein concentration was determined either spectrophotometrically or by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

(a) Spectrophotometric method: Protein concentration was determined following absorbance measurement at 279 nm using the value of specific extinction coefficient ($E^{1\%}_{1cm}$) as 6.67 and 5.31 for bovine serum albumin and human serum albumin, respectively (Peters, 1985).

(b) Method of Lowry *et al.* (1951): Increasing volumes (0.1-1.0 ml) of stock protein solution (0.5 mg/ml) were taken in series of tubes and the volume in each tube was made to 1.0 ml, if required, with 0.07 M sodium phosphate buffer, pH 7.4 containing 0.08 M NaCl. Then, 5.0 ml of freshly prepared copper reagent was added to all the tubes and the contents were mixed well. After 10 minutes of incubation at room temperature, 1.0 ml of diluted Folin-phenol reagent was added and vortexed. The tubes were then incubated for 30 minutes at room temperature and the color intensity was read at 700 nm against a suitable blank prepared in the same way as that of test solution except that instead of protein solution, 1.0 ml of buffer was taken. A calibration curve, thus obtained, between absorbance at 700 nm and amount of protein (mg) yielded the following straight line equation (Fig.7):

$$(\text{Absorbance})_{700\,\text{nm}} = 1.72\ (\text{amount of protein, mg}) + 0.087 \qquad (1)$$

Protein measurements were also performed in 50 mM tris-HCl buffer, pH 7.4 as well as 10 mM tris-HCl buffer, pH 7.4 containing 1% SDS. The above protocol was employed to obtain other straight line equations for protein in 50 mM tris-HCl buffer, pH 7.4 and 10 mM tris-HCl buffer, pH 7.4 containing 1% (w/v) SDS, respectively (Fig.7):

$$(\text{Absorbance})_{700\,\text{nm}} = 1.13\ (\text{amount of protein, mg}) + 0.003 \qquad (2)$$

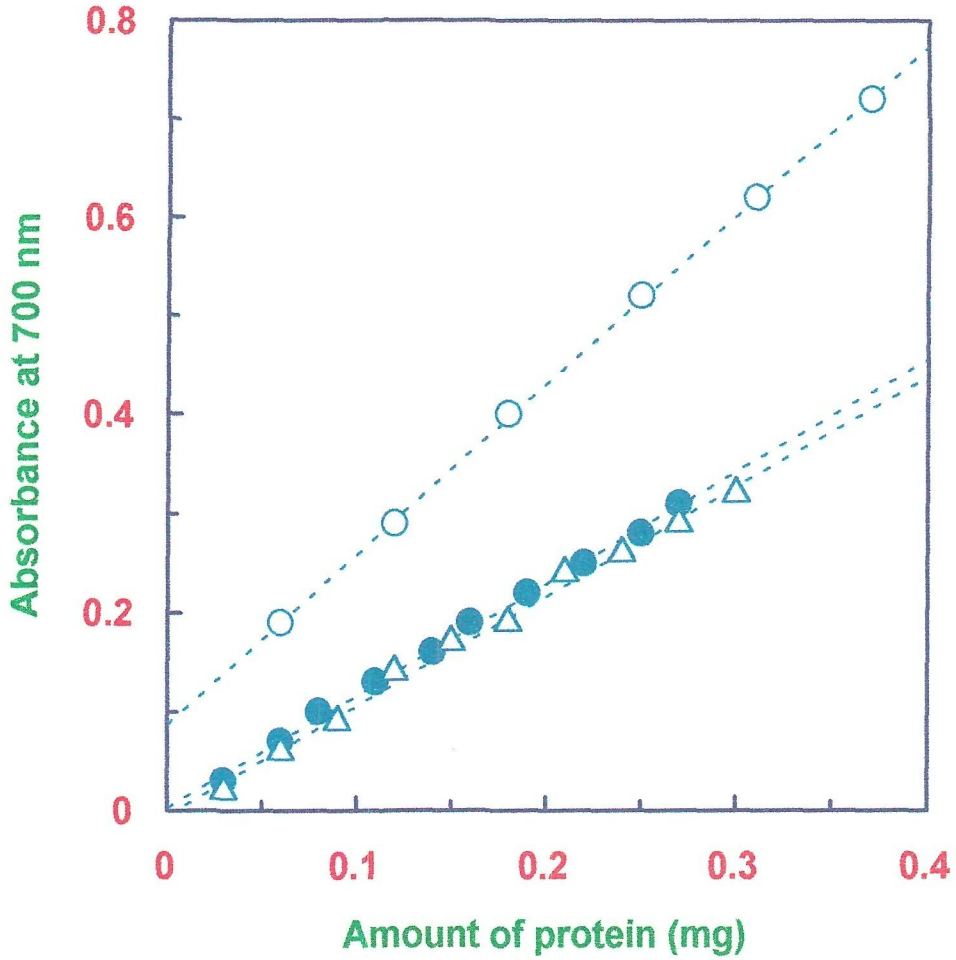


Figure 7. Standard curves for the determination of protein concentration by the method of Lowry *et al.* (1951) obtained with 0.07 M sodium phosphate buffer, pH 7.4 containing 0.08 M NaCl (○), 50 mM tris-HCl buffer, pH 7.4 (●) and 10 mM tris-HCl buffer, pH 7.4 containing 1% (w/v) SDS (△).

$$(\text{Absorbance})_{700\,nm} = 1.11\ (\text{amount of protein, mg}) + (-0.005) \quad (3)$$

For the estimation of erythrocyte membrane proteins by the method of Lowry *et al.* (1951), membranes were subjected to solubilization in 1% (w/v) SDS. To 0.1 ml of erythrocyte membrane suspension in 50 mM tris-HCl buffer, pH 7.4 was added 0.9 ml of 1.1% (w/v) SDS and incubated at 60°C for 30 minutes. After complete solubilization of membranes, the above protocol was followed and protein concentration was determined using equation (3).

4. Isolation of serum albumin

Serum albumin was isolated from human/goat blood according to the method described by Tayyab & Qasim (1990). Blood was centrifuged at 1500×g for 20 minutes to obtain plasma, which was brought to 2.26 M in ammonium sulfate by adding requisite volume of 4 M ammonium sulfate solution, pH 7.0. The mixture was kept for 12 hours at room temperature and then carefully diluted with water and with frequent additions of 0.5 N H_2SO_4 such that the final concentration of ammonium sulfate was reduced to 1.9 M and the pH to 4.2. After incubating it for 12 hours at room temperature, the precipitate was collected by centrifugation at 3000×g for 30 minutes. It was washed three times with 2.2 M ammonium sulfate solution, pH 4.2 and then dissolved in 0.07 M sodium phosphate buffer, pH 7.4 containing 0.08 M NaCl. The protein preparation, thus obtained, was extensively dialyzed against 6 litres of the desired buffer, either 0.07 M sodium

phosphate buffer, pH 7.4 containing 0.08 M NaCl or 50 mM tris-HCl buffer, pH 7.4 and stored at 4°C.

5. Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis of human serum albumin and goat serum albumin was performed in 8 % (w/v) polyacrylamide gel while electrophoresis of human erythrocyte membrane and its treated derivatives was performed in 10% (w/v) polyacrylamide gels, using 0.02 M tris-glycine buffer, pH 8.2, ionic strength 0.02 following the method of Laemmli (1970) under non-denaturing and denaturing conditions, respectively. Upto 5-8 μg for serum albumins and 50 μg of protein for erythrocyte membrane and their derivatives was applied in 20 μl of sample buffer, and a current of 3-4 mA per well was passed for nearly 2 hours.

(a) The gels were stained with a staining solution containing 0.25% (w/v) coomassie brilliant blue in 40% (v/v) methanol and 10% (v/v) acetic acid and destained with 10% (v/v) acetic acid solution containing 10% (v/v) methanol at 37°C.

(b) Fixing and staining with carbocyanine was performed by the method followed by King *et al.* (1976). The gels were fixed in 10-20 ml of 10-25% isopropanol and then washed exhaustively in 25% isopropanol for 18-36 hours with frequent changes or heated to 56°C for 1-2 hours to remove SDS. Staining method of Green *et al.* (1973) was employed where 5.0 ml of stock solution of carbocyanine (0.1% w/v in formamide) was diluted with 20 ml formamide, 100

ml isopropanol and 275 ml of 15 mM tris to give 400 ml. The pH was adjusted to 8.5 with NaOH or HCl. The gels were placed in 10-20 ml of working stain solution overnight in dark. The gels were destained in 10-20 ml of 10% isopropanol for 18-36 hours at 25°C.

In crosslinking experiments, unreduced membrane samples were prepared in the same way as described above but in the absence of 2-mercaptoethanol. The gels were stained for proteins with coomassie blue.

6. Determination of bilirubin concentration

Bilirubin concentration was determined by modified Jendrassik & Grof method (1938) [Fog's method (1958)] as "alkaline azobilirubin". The method involved the use of three reagents namely, I, II and III which were prepared in the following manner:

(i) **Preparation of reagent I :** Twenty grams of caffeine anhydrous, 30 gms of sodium benzoate and 50 gms of sodium acetate were dissolved in 400 ml of water and the mixture was warmed up to 50°C with continuous stirring until a clear transparent solution was obtained.

(ii) **Preparation of reagent II :** Working solution of reagent II was prepared fresh just before the experiment by adding 3 drops of reagent IIb (containing 500 mg of sodium nitrite in 100 ml of water) in 5.0 ml of reagent IIa (prepared by dissolving 0.5 gm of sulfanilic acid in 1.5 ml of concentrated hydrochloric acid followed by dilution with 100 ml of water).

(iii) Preparation of reagent III : Thirty grams of sodium hydroxide was dissolved along with 105 gm of sodium potassium tartarate in 300 ml of water.

Preparation of stock bilirubin solution

Stock solution of bilirubin was prepared by dissolving few crystals of bilirubin in 1N NaOH, containing 5 mM EDTA and diluting it to 10 ml with 0.06 M sodium phosphate buffer, pH 8.0. The concentration of bilirubin was determined spectrophotometrically using a molar absorption coefficient of 47,500 $M^{-1}cm^{-1}$ at 440 nm (Jacobsen & Wennberg, 1974). Bilirubin solution was stored in dark. All the spectral measurements were recorded in dim/yellow light to prevent undesired photodegradation of bilirubin and the bilirubin solution was used within an hour of preparation.

Procedure: Increasing volumes (0.1-1.0 ml) of stock bilirubin solution (90 μM) were added to different tubes and the final volume was made to 1.0 ml, if required, with buffer. This was followed by the addition of 2.0 ml of reagent I and 0.5 ml of reagent II, with gentle mixing. After 10 minutes of incubation at room temperature, 1.5 ml of reagent III was added to all the tubes and the contents were mixed well. Absorbance of the solution was read at 600 nm against buffer. For each bilirubin concentration, reagent II blank and bilirubin blank were prepared in the same way as described above except that buffer was used instead of reagent II and bilirubin in these blanks, respectively. Absorbance of diazotised bilirubin solution was determined by subtracting the absorbance values of reagent II blank and bilirubin blank from the absorbance of bilirubin solution.

Data were plotted as absorbance at 600 nm versus bilirubin concentration, which yielded the following straight line equation (Fig.8):

$$(\text{Absorbance})_{600nm}=0.015\ (\text{amount of bilirubin, nmoles}) + (-0.0064) \quad (4)$$

Presence of either serum albumin or 1% (w/v) SDS in the bilirubin stock solution and the use of 38 mM sodium carbonate solution containing 5 mM EDTA, pH 11.0 instead of 0.06 M sodium phosphate buffer, pH 8.0 did not affect the value of slope of the standard curve (Fig.8). In SDS containing samples, turbidity caused by SDS was removed by centrifugation at 16,000×g for 20 minutes.

For experiments involving metal ions, EDTA was eliminated from the solutions. Therefore, bilirubin solution was prepared in 38 mM sodium carbonate solution, pH 11.0. A calibration curve in the presence of $CaCl_2$ (1,2 and 3 mM) was also plotted in the same way as above (Fig.8). The increasing concentration of $CaCl_2$ did not change the slope of the calibration curve. In the presence of metal ions, the straight line relationship is:

$$(\text{Absorbance})_{600nm}= 0.012\ (\text{amount of bilirubin, nmoles}) + (-0.0146) \quad (5)$$

7. Inorganic phosphorus estimation

(a) Inorganic phosphorus was determined by the method of Fiske &

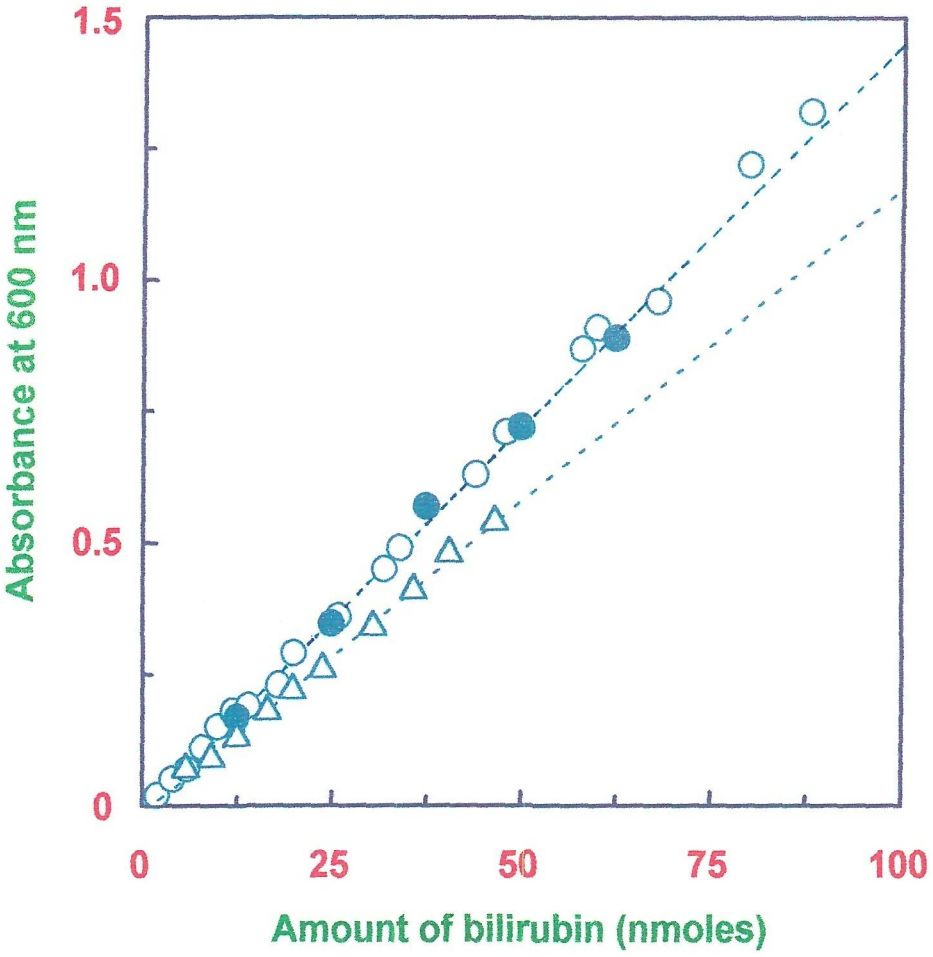


Figure 8. Standard curves for the determination of bilirubin concentration by Fog's method (1958) obtained with 1 N sodium hydroxide containing 5 mM EDTA (○), 38 mM sodium carbonate solution, pH 11.0 containing 1% (w/v)SDS (●) and 38 mM sodium carbonate solution , pH 11.0 containing 3 mM $CaCl_2$ (△).

Subbarow (1925). Different volumes of stock solution, prepared by dissolving 16.67 mg of di-sodium hydrogen phosphate in 100 ml of water (equivalent to 37.6 μg of inorganic phosphorus per ml of this solution), were taken in the range of 0.1-1.0 ml in different tubes and the volume in each tube was adjusted to 5.0 ml with water. Then 0.2 ml of 5% (w/v) ammonium molybdate was added to all the tubes and mixed well. It was followed by the addition of 0.2 ml of 0.1% (w/v) ascorbic acid. The contents were mixed well and the tubes were incubated at 80°C for 30 minutes, then cooled under tap water and the absorbance was read at 700 nm against an appropriate blank. Data were plotted as absorbance at 700 nm versus amount of inorganic phosphorus, which yielded the following straight line equation (Fig.9):

$$(\text{Absorbance})_{700nm} = 0.025\ (\text{amount of inorganic phosphorus, } \mu g) + 0.029 \qquad (6)$$

(b) Inorganic phosphorus measurement was also performed by the method of Ames & Dubin (1960). Increasing volumes of stock solution prepared by dissolving 5 mg of di-sodium hydrogen phosphate in 5 ml of water (equivalent to 225 μg of inorganic phosphorus per ml of this solution), were taken in the range of 5-50 μl in different tubes and 0.18 ml of 10% magnesium nitrate dissolved in absolute alcohol was added to each tube. Each of these tubes was ignited to get white ash and then 1.8 ml of 0.5 N HCl was added to all tubes. The contents were mixed well with a vortex and heated in a boiling water bath for 15 minutes for

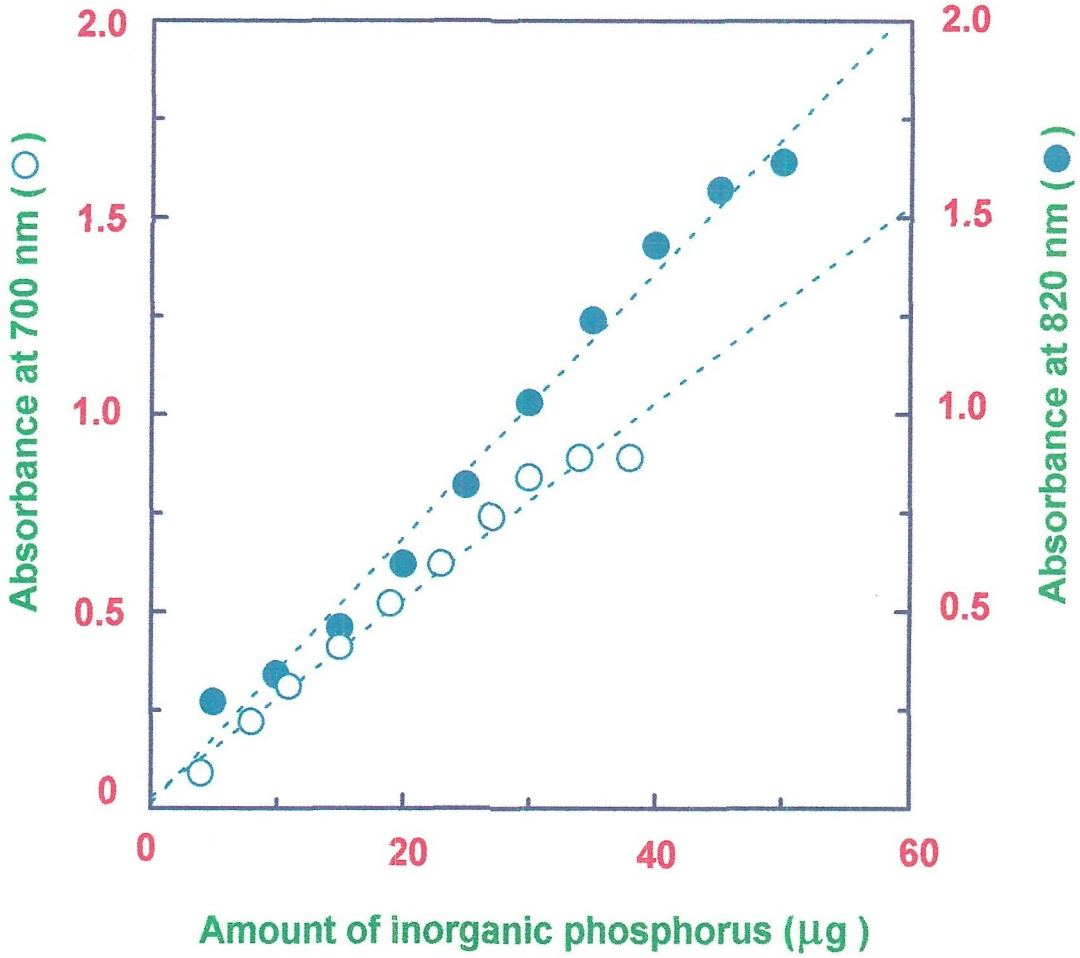


Figure 9. Standard curves for the detemination of inorganic phosphorus content by the method of Fiske & Subbarow (1925) (○) and Ames & Dubin (1960) (●).

complete digestion. The tubes were cooled under tap water and 4.2 ml of ammonium molybdate-ascorbic acid solution (6:1), prepared fresh, was added to all the tubes. Absorbance was read at 820 nm. Data were plotted as absorbance at 820 nm versus amount of inorganic phosphorus, which yielded the following straight line equation (Fig.9):

$$(\text{Absorbance})_{820nm} = 0.034\ (\text{amount of inorganic phosphorus, } \mu g) + 0.013 \qquad (7)$$

For the determination of total inorganic phosphorus of erythrocyte membranes, initially the organic matter was destroyed by heating the membranes (0.2 ml suspension in 50 mM tris-HCl buffer, pH 7.4) at 180°C for 2 hours with 1.0 ml of 70% (v/v) perchloric acid and then adjusting the volume to 1.0 ml with water. The inorganic phosphorus was determined in the same way as described above using equation (6).

8. Sialic acid estimation

Thiobarbituric acid assay, given by Warren (1959) was employed for the determination of sialic acid content using N-acetylneuraminic acid as the standard. The following reagents were used in this assay:

(a) 0.2 M sodium meta-periodate in 9 M orthophosphoric acid

(b) 10% (w/v) sodium arsenite in a solution of 0.5 M sodium sulfate and 0.1N sulfuric acid

(c) 0.6% (w/v) thiobarbituric acid in a solution of 0.5 M sodium sulfate.

All the above solutions were stored at room temperature and used within a month.

Procedure: To a volume ranging from 20-200 µl of stock solution, prepared by dissolving 3.8 mg of N-acetylneuraminic acid in 50 ml of water (equivalent to 0.25 µmole of N-acetylneuraminic acid per ml of this solution), was added water, if required, to make up the volume to 0.2 ml. Then 0.1 ml of periodate solution was added. After mixing, the contents were kept at room temperature for 20 minutes. One ml of arsenite solution was added to all the tubes and the contents were shaken well until a yellow color, first formed, disappeared. Then, 3.0 ml of thiobarbituric acid reagent was added and the tubes were heated at 100°C for 15 minutes by keeping them in vigorously boiling water bath. The tubes were then cooled under tap water and the entire solution was added to an equal volume of cyclohexanone. The tubes were shaken well and centrifuged at 1500×g for 5 minutes. The upper pink layer was aspirated with Pasteur pipette and the absorbance was read at 550 nm against the blank prepared in the same way but without N-acetylneuraminic acid. The data were plotted as absorbance at 550 nm versus µmoles of N-acetylneuraminic acid (NANA) (Fig.10), which yielded the following straight line equation:

$$(\text{Absorbance})_{550nm} = 12.72\ (\text{amount of N-acetylneuraminic acid, µmoles}) + 0.016 \qquad (8)$$

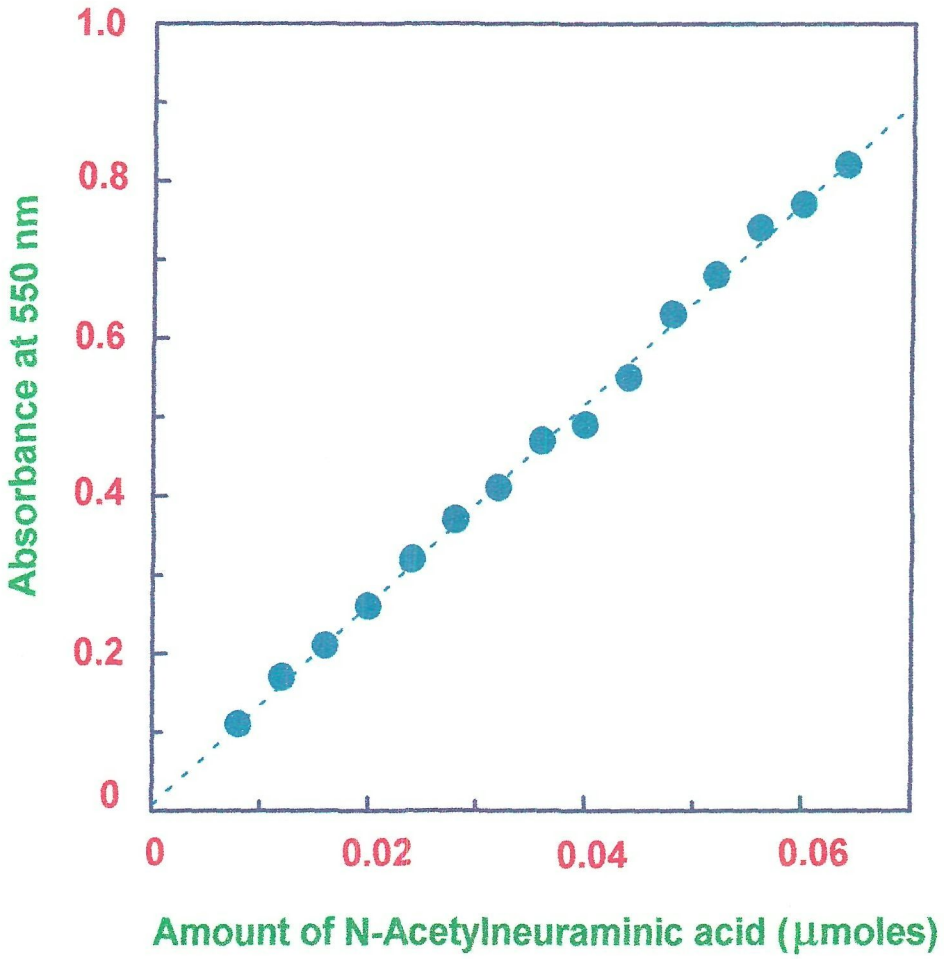


Figure 10. Standard curve for the determination of sialic acid content following the method of Warren (1959).

For the determination of sialic acid content of erythrocyte membranes, sialic acid was liberated from membranes by mild acid hydrolysis in 0.1N sulfuric acid at 80°C for 1 hour. Then, the solution was cooled to room temperature, centrifuged at 16,000×g for 20 minutes and sialic acid content was determined in the same way as described above using equation (8).

9. Carbohydrate estimation

Orcinol method was employed for the determination of carbohydrate concentration using glucose (0.2 mg/ml) as the standard (Svennerholm, 1956). To 1.0 ml of solution containing 20-200 µg glucose was added 200 µl of orcinol reagent (prepared by dissolving 150 mg orcinol in 10 ml of 30% (v/v) sulfuric acid) followed by the addition of 2.5 ml of 60% (v/v) sulfuric acid. The contents were rapidly mixed and incubated at 80°C for 20 minutes. The tubes were then cooled and left for 45 minutes in the dark. The absorbance was read at 470 nm against a blank prepared I the same way but substituting water for sugar solution. The data, thus obtained, were plotted as absorbance at 470 nm versus the amount of glucose (mg) which yielded the following straight line equation (Fig.11):

$$(\text{Absorbance})_{470\text{nm}} = 3.67\ (\text{amount of glucose, mg}) + 0.018 \qquad (9)$$

The carbohydrate content of erythrocyte membranes was determined in the same way by subjecting the erythrocyte membranes directly to the orcinol

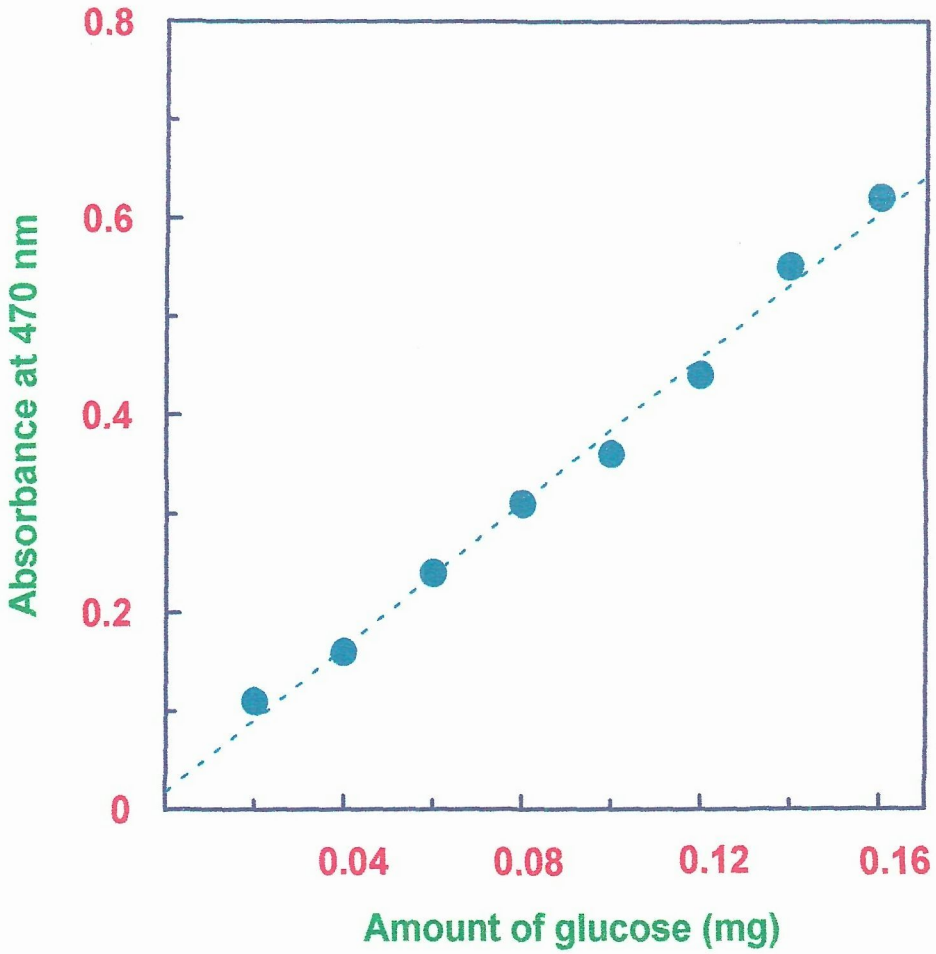


Figure 11. Standard curve for the determination of carbohydrate content by orcinol method (Svennerholm, 1956).

reaction.

10. Choline estimation

The choline content was determined following the procedure of Kates & Sastry (1969). Varying volumes of stock choline chloride solution (1.15 g/litre) ranging from 4-20 μmoles of choline in a volume of 3.0 ml were taken in a graduated 15 ml centrifuge tube and a few drops of phenolphthalein solution (1% in ethanol-water in 1:1, v/v) were added. The solution was made alkaline by the addition of 2N NaOH and diluted with water to 5 ml. To this, 2.5 ml of ammonium reineckate solution (2% in methanol) was added and the contents were swirled in a vortex mixer and kept at 5°C for 2 hours.

Pink precipitate of choline reineckate is spun down in a centrifuge at 16,000×g for 20 minutes, washed twice with 1.0 ml portions of n-propanol and then dissolved in 5.0 ml of acetone, centrifuged, decanted and read at 526 nm against a blank where choline chloride was substituted with water. The data, thus obtained, were plotted as absorbance at 526 nm against the amount of choline yielding the following straight line equation (Fig.12):

$$(\text{Absorbance})_{526nm} = 0.18\ (\text{amount of choline, } \mu\text{moles}) + 0.031 \qquad (10)$$

11. Preparation of erythrocyte suspension

Human/goat erythrocytes were collected by centrifugation of blood at 1000×g for

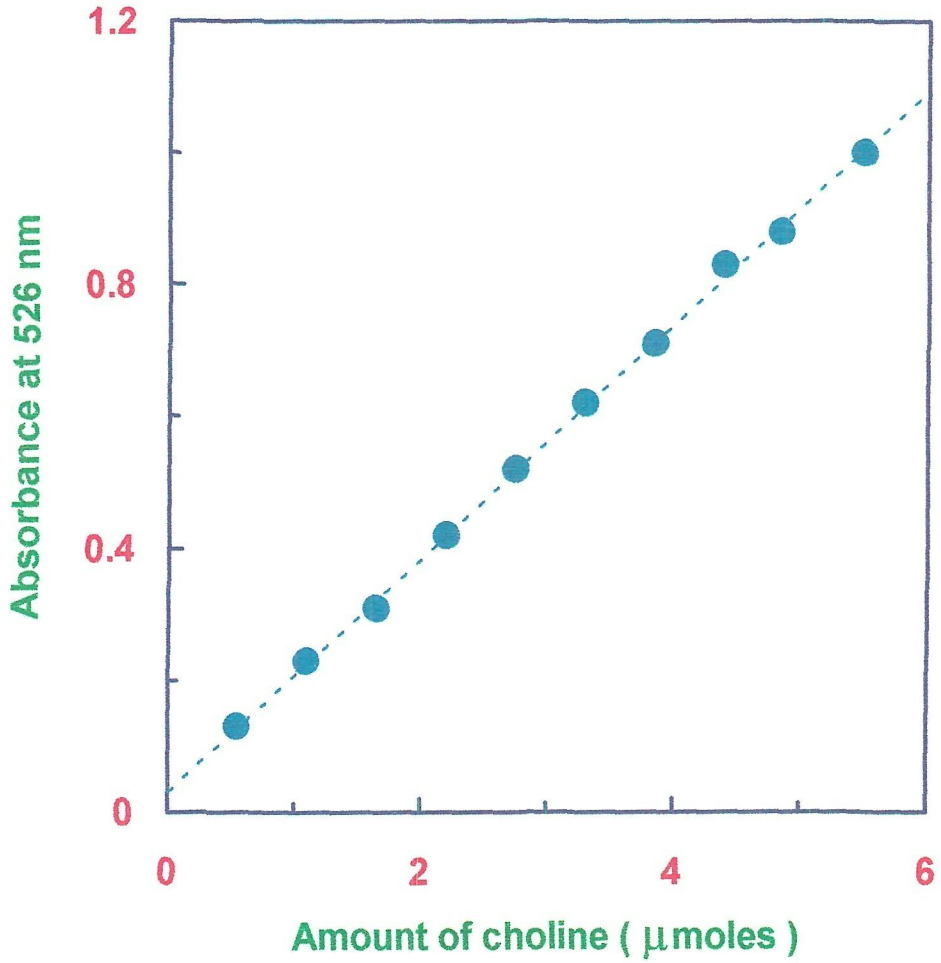


Figure 12. Standard curve for the determination of choline content by the method of Kates & Sastry (1969).

20 minutes at 4°C. Plasma and "buffy coat" were removed by careful aspiration and cells were then resuspended in 50 mM tris-HCl buffer, pH 7.4 containing 100 mM NaCl or 50 mM sodium phosphate buffer, pH 7.4/8.0 containing 150 mM NaCl followed by triple washing with the same buffer. After each step of centrifugation, the surface of the pellet was thoroughly aspirated. The packed washed cells were diluted with an equal volume of the same buffer to obtain 50% hematocrit value.

12. Preparation of erythrocyte membrane suspension

Erythrocyte membranes were prepared in the same way as described by Palfrey & Waseem (1985). Erythrocyte suspension of 50% hematocrit value was hemolysed with 10 volumes of cold 10 mM tris-HCl buffer, pH 7.4 containing 0.01 mM EDTA and 0.01 mM PMSF followed by gentle swirling and centrifugation at 16,000×g for 20 minutes at 4°C. The dark red supernatant was removed carefully by gentle aspiration. Each tube was tilted and rotated to allow the loose ghost pellet to slide off to another tube, leaving the tightly packed "buttons" at the bottom of the tube. This minimized the contamination of the ghost with proteinases as suggested by Fairbanks *et al.* (1971). The ghost pellet, thus obtained, was washed several times with the same buffer followed by centrifugation at 16,000×g for 20 minutes at 4°C until membranes were free from hemoglobin. After final washing, the erythrocyte membrane pellet was resuspended in the same hemolysing buffer such that the total volume of

membrane suspension was equal to the volume of erythrocyte suspension of 50% hematocrit value initially taken for hemolysis. This suspension was stored at 10°C and washed once with 50 mM tris-HCl buffer, pH 7.4 prior to use and the volume was restored to the initial volume taken with the same buffer. However, erythrocyte membrane suspension stored in 10 mM tris-HCl buffer, pH 7.4 could be used within five days when kept at 10°C.

Density separation of erythrocytes into different age groups was based on the method of Murphy (1973). Fresh blood from donor (B-Rh+ve) was centrifuged in graduated centrifuge tubes at 1000×g for 10 minutes at 30°C, the plasma was retained and the "buffy coat" was discarded carefully avoiding loss of erythrocytes. The cells were then washed by adding back their own plasma and recentrifuged as above. The plasma and "buffy coat" were again discarded carefully. The erythrocytes, which were at about 90 % hematocrit, then were spun at 30,000×g for 1 hour at 30°C. Any excess plasma and buffy coat were removed and discarded taking care of loss of erythrocytes. Fractions of the cells were collected by volume from the top to the bottom of the tube representing the top 10 %, two middle fractions of 40 % each, and the bottom 10 %. These four fractions were young (top), intermediate (two middle) and old (bottom) erythrocytes, respectively. These fractions were washed with 50 mM tris-HCl buffer, pH 7.4 containing 150 mM NaCl and were then hemolysed using the same hemolysing buffer as that used for the preparation of erythrocyte membranes described earlier.

Different procedures were adapted for using these membranes in different experiments.

(a) For experiments involving the effect of pH and temperature on human erythrocyte membranes, the prepared erythrocyte membrane suspension was divided into aliquots, washing each of them with respective 50 mM tris-HCl buffer of different pH i.e., 7.8, 7.6, 7.4, 7.2, 7.0 and mixed with a volume of buffer equivalent to the volume of the aliquot taken initially.

(b) Different membrane vesicles such as sealed, heterogeneous and inside-out vesicles were prepared from the unsealed membrane vesicles following the method of Steck & Kant (1973) with little modification.

Unsealed erythrocyte membranes were obtained after hemolysing erythrocyte suspension of 50% hematocrit value with 5 mM sodium phosphate buffer, pH 7.4 in ratio of 1:10 followed by centrifugation at 16,000×g for 20 minutes at 4°C and washing as described above.

For the preparation of sealed membrane vesicles, the unsealed membrane vesicles were resuspended to a final volume of 40 ml in 5 mM sodium phosphate buffer, pH 7.4 containing 150 mM NaCl and incubated at 37°C for 40 minutes followed by centrifugation at 16,000×g for 20 minutes at 4°C. The pellet, thus obtained, was collected and used as such in further studies.

Heterogeneous membrane vesicles were prepared by washing the unsealed membranes with 5 mM sodium phosphate buffer, pH 7.4 containing 10^{-4} M $MgSO_4$ followed by centrifugation at 16,000×g for 20 minutes at 4°C. The pellet

was resuspended in the same buffer to a final volume of 40 ml and incubated at 37°C for 40 minutes followed by centrifugation at 16,000×g for 20 minutes at 4°C.

Inside-out membrane vesicles were prepared from the unsealed membrane vesicles after washing them with 0.5 mM sodium phosphate buffer, pH 8.2 followed by centrifugation at 16,000×g for 20 minutes at 4°C. The pellet, thus obtained, was resuspended in 40 volumes of the same buffer and kept for 18 hours on ice. The membranes were pelleted at 16,000×g for 20 minutes at 4°C and resuspended again in the same buffer. Then contents were homogenized on a vortex, passed three to four times through No. 27 gauge needle followed by centrifugation at 16,000×g for 20 minutes at 4°C and the pellet collected.

The pellets of all types of membrane vesicles were finally suspended in 5 mM sodium phosphate buffer, pH 7.4 and used immediately for bilirubin binding studies.

(c) Sealed and human serum albumin (HSA)-entrapped sealed human erythrocyte membranes were prepared by employing the buffer system used by Steck & Kant (1973) with a little modification. Erythrocytes prepared, as above were hemolysed in cold 5 mM sodium phosphate buffer, pH 8.0 (5P8) in a ratio of 1:40. The membranous ghosts were pelleted by centrifugation at 16,000×g for 20 minutes at 4°C followed by subsequent washes with the same buffer as described above. These washes were performed till milky white morphologically intact unsealed membranes were obtained.

To 5.0 ml of these unsealed membranes was added 3.0 ml of 20 % HSA solution prepared in 5 mM sodium phosphate buffer, pH 8.0 containing 10^{-4} M magnesium sulfate (5P8-$MgSO_4$). Another set was prepared where HSA was substituted with 5P8-$MgSO_4$. The two suspensions were then dialyzed extensively (overnight) against the same buffer with frequent changes. Under these conditions, membranes resealed and trapped albumin inside. Both the suspensions were washed with the same buffer and pelleted by centrifugation at 16,000×g for 20 minutes at 4°C. The supernatant was checked after every wash for protein content. Washing was performed till no protein content was detected in the supernatant and the volume was made upto 8.0 ml with the same buffer. This resulted in the formation of HSA-entrapped sealed human erythrocyte membranes and sealed human erythrocyte membranes, respectively. These preparations were maintained in the same buffer (5P8-$MgSO_4$) and used immediately for further studies. In order to check the feasibility of resealing, sealed membranes prepared in the same way as described above were incubated with the similar amount of albumin used earlier and kept overnight under the same conditions mentioned. The preparation was then centrifuged at 16,000×g for 30 minutes and washed with 5P8-$MgSO_4$ till the protein content in the supernatant becomes negligible. These membranes were used as such for bilirubin binding studies employing CD spectroscopy.

The amount of HSA trapped inside the sealed membranes was determined from the difference of protein content of HSA-entrapped membranes and sealed

membranes after digesting both the preparations in 1% SDS and estimating protein content by the method of Lowry *et al.* (1951).

13. Enzymatic and other treatments of erythrocyte membranes

(a) Phospholipase C treatment of erythrocyte membranes was performed according to the method of Sato *et al.* (1987). To 0.5 ml of membrane suspension (~ 4 mg protein/ml) in 50 mM tris-HCl buffer, pH 7.4 was added 4.25 ml of 0.1 M tris-HCl buffer, pH 7.4, 50 µl of 1.0 M $CaCl_2$ and 200 µl of the enzyme solution (0.15 mg/ml) and the mixture was incubated for different time periods at 37°C. After a desired period of incubation, all the contents were centrifuged at 16,000×g for 20 minutes at 4°C and the released phosphate (Pi) in the supernatant was measured by the method of Fiske & Subbarow (1925). The treated membrane pellet was washed once with cold 50 mM tris-HCl buffer, pH 7.4 containing 10 mM EDTA followed by centrifugation at 16,000×g for 20 minutes at 4°C. This reaction mixture for the binding assay became turbid during the incubation with phospholipase C and turbidity increased with time.

(b) Phospholipase D digestion of erythrocyte membranes was carried out following the method of Sato *et al.* (1987). To 0.5 ml of erythrocyte membrane suspension was added 1.4 ml of 0.1 M tris-HCl buffer, pH 7.4 and 100 µl of enzyme solution (9.3 units). The mixture was incubated for different time periods at 37°C. After the desired period of incubation, the contents were centrifuged at

16,000×g for 20 minutes at 4°C. The supernatant was assayed for released choline by the method of Kates & Sastry (1969).

(c) Ghost suspension (0.5 ml) was digested with neuraminidase (2 units) for different time periods at 37°C in 1.0 ml of 0.1 M tris-HCl buffer, pH 7.4 (Sato *et al.*, 1987). The mixture was centrifuged at 16,000×g for 20 minutes at 4°C after desired incubation and the supernatant containing released sialic acid was collected and subjected to sialic acid estimation as described by Warren (1959).

(d) The percentage accessibility of sialic acid in different membrane vesicles preparations to neuraminidase was determined by the method of Steck & Kant (1973) after treating the membranes containing 100-300 µg protein in 50 µl with neuraminidase (0.1 mg/ml) for 30 minutes at 25°C both in the presence and absence of 0.2% (v/v) Triton X-100. The released sialic acid was determined directly by the method of Warren (1959).

(e) Method of Steck *et al.* (1971) was used for tryptic digestion of erythrocyte membrane which was performed by incubating 0.5 ml of membrane suspension with 1.0 ml of 2.5 mM tris-HCl buffer, pH 8.1 containing 5 mg of solid trypsin at 37°C and the mixture was shaken gently to dissolve the enzyme completely. After the desired time of incubation, the mixtures were centrifuged at 16,000×g for 20 minutes at 4°C and the released glycopeptides in the supernatant were measured for carbohydrate content according to Svennerholm (1956).

(f) Membrane suspension (0.5 ml) was incubated with different volumes of 1.0 M $CaCl_2$ in 50 mM tris-HCl buffer, pH 7.4, making the total volume of 1.5

ml, for about 30 minutes at 37°C. Unbound metal ions were removed by centrifugation of the membranes at 16,000×g for 20 minutes at 4°C.

Treated membranes stated above (a-f) were washed with cold 50 mM tris-HCl buffer, pH 7.4 twice followed by centrifugation at 16,000×g for 20 minutes at 4°C and the final pellet, thus obtained, was resuspended in the same buffer after making up the volume to 1.0 ml. These preparations were directly used for bilirubin binding experiments.

(g) Various other treatments of human erythrocyte membranes were carried out by the procedure described by Kahlenberg (1972). Membranes (1.0 ml in 50 mM tris-HCl buffer, pH 7.4) were treated separately with DMMA (20 mg/ml of incubation mixture), 0.1 mM EDTA (pH 8.0), 20 mM LIS, 1.0 M NaI and 1.0 mM NaOH (pH 11.5) in a total volume of 4.0 ml for 1 hour at 37°C. The supernatant and pellet fractions were separated by centrifugation at 16,000×g for 20 minutes at 4°C. The pellet, so obtained, was washed twice, with either 50 mM tris-HCl buffer, pH 7.4 or 5 mM sodium phosphate buffer, pH 7.4 and suspended in 1.0 ml of the same buffer.

(h) Oxidative cross-linking of erythrocyte membrane proteins was carried out according to the method of Kahlenberg (1972). To 1.0 ml of membrane suspension in 50 mM tris-HCl buffer, pH 7.4 was added 1.0 ml of the solution containing 100 μM o-phenanthroline and 20 μM $CuSO_4$. The total volume was made to 4.0 ml with the same buffer and incubated for 1 hour at 37°C. Half of these samples received 100 mM 2-mercaptoethanol after 30 minutes of

incubation and further incubated for additional 30 minutes. Membrane pellets obtained by centrifugation were washed in the same way as described above and used directly for bilirubin binding experiments.

14. Extraction and analysis of phospholipids

Untreated and phospholipase C-treated membranes were subjected to phospholipid extraction by the method of Gier & Deenen (1961). To 1.0 ml of membrane suspension was added 5.0 ml of isopropanol with intermittent shaking. After 2 hours, 2.0 ml of chloroform was added and the suspension was kept overnight at 10°C. The supernatant, obtained by centrifugation at 16,000×g for 20 minutes, was dried under reduced pressure in a rotary evaporator, washed thrice with benzene and finally dissolved in 500 µl of chloroform.

The phospholipids, thus obtained, were separated by two-dimensional thin-layer chromatography following the method of Dittmer & Wells (1969) on silica impregnated aluminium sheets (20×20 cms) using a pair of solvents introduced by Rouser *et al.* (1969). Phospholipid extracts (1-5 µg of lipid phosphorus) are applied in a 1 cm diameter spot in one corner located 3 cm from each edge of the silica impregnated aluminium sheet. Application of too much lipid in a very small area at the origin usually leads to spot elongation and streaking. The lipids were applied in a 20-30 µl volume at one time. Ascending chromatography was carried out at 23°C in a constant temperature room. The sheet was placed in a developing jar containing 105 ml of solvent I (chloroform, methanol, 28%

ammonia in a ratio 65:35:5, respectively). The chamber was sealed with a lid and the chromatogram is developed. After the first run, the chromatogram was dried for 30 minutes at room temperature and the second dimension was run at right angles to the direction of the first solvent in a sealed jar containing 104 ml of solvent II (chloroform, methanol, acetone, acetic acid and water in the ratio 60:12:24:12:6, respectively).

Detection of phospholipid spots:

After the chromatogram was developed, it was dried at room temperature for 30 minutes and finally placed in a jar of iodine for the detection of the phospholipid spots. Since spots fade on drying, it was found useful to circle the spots with a pencil.

Each spot produced was scraped off and collected in separate tubes. It was then eluted with 5.0 ml of chloroform-methanol mixture (1:1) for 2 hours and the eluent was evaporated in a boiling water bath. Each sample was subjected to inorganic phosphorus estimation by the method of Ames & Dubin (1960). The phospholipid content was estimated by multiplying the total inorganic phosphorus content by 25 as suggested by Berg (1969).

15. Bilirubin binding experiments

Bilirubin solution was prepared by dissolving a few crystals of bilirubin in 38 mM sodium carbonate solution containing 5 mM EDTA, pH 11.0. The concentration of bilirubin solution was determined by Fog's method (Fog, 1958).

However, for bilirubin binding study with $CaCl_2$-treated membranes, EDTA was excluded from the solubilization medium. Following procedures were adapted for studying the binding of bilirubin to erythrocyte membranes.

(a) Binding of bilirubin to erythrocyte membranes at different pH was studied by addition of different volumes (20-250 μl) of stock bilirubin solution to 1.0 ml of albumin solution of known concentration in 50 mM tris-HCl buffer having pH X (where X= 7.0, 7.2, 7.4, 7.6 or 7.8) to get different bilirubin/albumin molar ratios, B/As (0.5-3.0) and the volume was made up to 1.25 ml with 50 mM tris-HCl buffer having same pH as that of albumin solution. Then, 250 μl of erythrocyte membrane suspension in the same buffer was added and the tubes were incubated for 30 minutes at 37°C after gentle shaking. In another set of experiments, both the bilirubin and albumin concentrations were varied to obtain a constant B/A (bilirubin concentration varying from 100-400 μM).

(b) Effect of temperature was studied by pre-incubating the membrane suspension in 50 mM tris-HCl buffer, pH 7.4 and bilirubin/albumin mixture of different B/As, independently at different temperatures (i.e., 7°C, 20°C, 27°C, 37°C, 40°C, 50°C and 60°C) for 30 minutes followed by mixing of bilirubin/albumin solution with membrane suspension and further incubation for 30 minutes at their respective temperatures.

(c) Binding of bilirubin to different erythrocyte membrane vesicles was studied by taking different volumes (40-400 μl) of albumin stock solution in 5 mM sodium phosphate buffer, pH 7.4 and adding increasing volumes (40 - 250

μl) of stock bilirubin solution to achieve a B/A of 2:1 and the volume in each tube was made up to 1.0 ml with the same buffer. Then 500 μl of erythrocyte membrane vesicles (4.7 mg protein per ml) was added and the tubes were incubated for 30 minutes at 37°C after gentle shaking.

(d) Binding of bilirubin to erythrocyte membranes (untreated/treated) was studied by incubating 1.0 ml of the membrane preparation in a final volume of 1.5 ml containing 250 μl of bilirubin (150 nmoles for experiments with phospholipase C and $CaCl_2$-treated membranes and 225 nmoles for other treated membranes) and 250 μl of albumin solution in 50 mM tris-HCl buffer, pH 7.4 to achieve a B/A of 2.0. The mixture was incubated for 30 minutes at 37°C.

(e) Binding of bilirubin to native and protein-depleted membranes was studied in 50 mM tris-HCl buffer, pH 7.4 after incubating these membranes (0.5 ml) with bilirubin (150 μM) in a total volume of 4.0 ml for 30 minutes at 37°C.

(f) Binding of bilirubin to sealed and HSA-entrapped sealed erythrocyte membranes was studied by CD spectroscopy. The bilirubin binding experiments were performed in 5P8-$MgSO_4$. The bilirubin solution was prepared by dissolving a few crystals of bilirubin in 1.0 ml of 38 mM sodium carbonate solution, pH 11.0 and immediately diluted with 5P8-$MgSO_4$. The spectral measurements were made after incubating 1.0 ml of membrane preparations (3.8 mg/ml of sealed and HSA-entrapped sealed) with 1.0 ml of bilirubin solution (150 μM) for 1 hour at 37°C. The spectra were recorded under dim/yellow light. All these experiments were performed at least thrice.

Bilirubin-loaded membranes obtained by centrifugation at 16,000×g for 20 minutes at 4°C in each of the above experiments, were washed 3-4 times with the same buffer used in their experimental protocol followed by centrifugation at 16,000×g for 20 minutes at 4°C until the last supernatant was devoid of yellow color. The membrane-bound bilirubin was estimated in the same way as described earlier (Tayyab & Ali, 1999) which is based on the determination of bilirubin as "azobilirubin" using Fog's method (1958) after SDS solubilization of membranes. The method is sensitive to measure a minimum amount of 3 nmoles of bilirubin in a given sample of 1.0 ml.

Bilirubin-bound membranes were solubilized in 1% (w/v) SDS (to a final volume of 1.5 ml) by incubating the contents at 60°C for one hour and Fog's reaction was carried out with 1.0 ml of the solubilized membranes to determine bilirubin concentration. The turbidity formed due to SDS was removed by centrifugation at 6000×g for 20 minutes.

Statistical analysis of the data included calculations of dispersion. Difference of means were tested for significance using two-tailed 't' test. The 't' values were used to calculate the P-value.

16. Drug binding studies

Ceftriaxone (29.8 mg) and sodium salicylate (0.72 mg) were dissolved in 5.0 ml of 5P8-$MgSO_4$. These solutions were protected from light and used within one hour. Binding of drug to sealed and HSA-entrapped sealed erythrocyte

membranes was studied both in the absence and presence (300-1200 μM) of drugs. To the incubation mixture, prepared above for bilirubin binding studies, was added 1.0 ml of 5P8-MgSO4 or drug (ceftriaxone/sodium salicylate). After incubation for 1 hour at 37°C, the CD spectral changes were observed.

17. Circular dichroism (CD) spectroscopy

CD spectra (of native, treated, sealed and HSA-entrapped membranes) in the absence as well as presence of bilirubin were recorded on a Jasco-spectropolarimeter, model J-720, equipped with a microcomputer. The instrument was calibrated with d-10 camphorsulfonic acid. All measurements were carried out at 25°C with the help of a thermostatically controlled cell holder attached to a Neslab RTE-110 circulating water bath with an accuracy of ±0.1°C. Spectra were collected at a scan speed of 20 nm/min and with a response time of 1 second. Each spectrum was the average of four scans. Far-UV CD spectra were taken in the wavelength range of 200-250 nm using quartz cuvettes of 1 mm path-length. The bilirubin and protein concentrations in these samples were 150 μM and 0.51 mg in a volume of 4.0 ml incubation mixture, respectively. CD values were transformed into molar ellipticity $[\theta]_\lambda$ using the following relationship:

$$[\theta]_\lambda = \theta \,.\, MRW / 10 \,.\, l \,.\, c \qquad (11)$$

where, θ is the observed ellipticity in degrees, c is the protein concentration in g/cm^3, l is the pathlength in cm and MRW is mean-residue weight taken as 114.3 g/mol (Chen *et al.*, 1974).

The visible-range CD spectra were measured in the wavelength range of 350-550 nm at 25±0.1°C using a cell holder of 10 mm pathlength. The spectra were collected at a scan speed of 500 nm/min with a response time of 1second. Each spectrum was the average of three scans. The bilirubin and protein concentrations used in this region were 150 μM and 1.0 mg in a volume of 3.0 ml incubation mixture, respectively.

Results & Discussion

RESULTS AND DISCUSSION

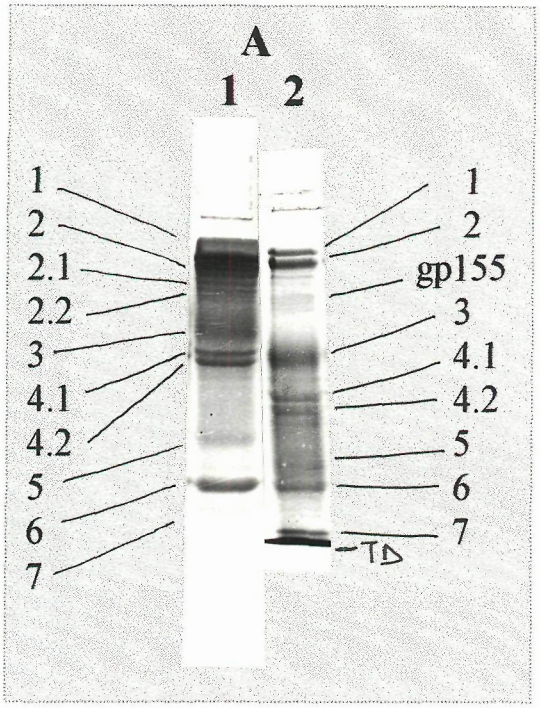
Isolation of serum albumin

Serum albumins of human and goat were isolated as described in the experimental section. About 2 grams of protein was obtained from 100 ml of the plasma. These preparations were found to be homogeneous as they gave a single symmetrical peak on 8 % polyacrylamide gel (Figure 13) showing a fair degree of charge homogeneity.

Electrophoretic pattern of human and goat erythrocyte membranes

Figure 14A (1) shows the electrophoretic pattern of human erythrocyte membrane polypeptides as visualized by coomassie dye on 10 % polyacrylamide gels in 1 % sodium dodecyl sulfate. The electrophorogram represents the six major bands constituting two thirds of the protein staining profile as given by Fairbanks *et al.* (1971). These bands were numbered according to Fairbanks *et al.* (1971) as Components I and II (mol wt. approx. 250,000) which form a slow moving doublet while Component III predominates with mol wt. 89,000. Then there is Component IV (mol wt 77,500), Component V (41,000) and Component VI (mol wt. 36,200). Fairbanks *et al.* (1971) have divided these components into two classes, components I, II, V and VI compose the class of proteins related to membrane by possible ionic bonds. The other class includes components III, IV and sialoglycoproteins, and these polypeptides are tightly bound to membrane. Goat erythrocyte membrane stained with coomassie blue (Figure 14A, 2) shows

Figure 13. Electrophoretic pattern (Native-PAGE) of goat serum albumin (well 1) and human serum albumin (well 2) on 8% polyacrylamide gel under non-denaturing conditions. About 10 μg of goat serum albumin and 30 μg of human serum albumin protein was loaded into each well, respectively. TD represents the position of tracking dye.



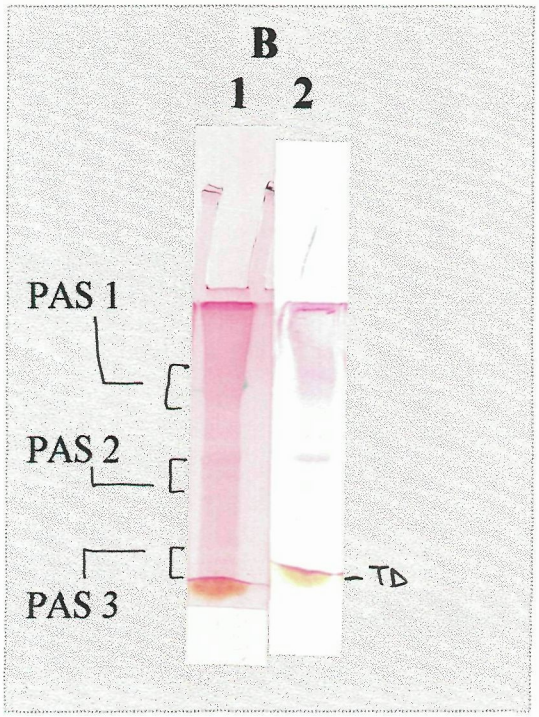


Figure 14. Electrophorogram (SDS-PAGE) of human erythrocyte membrane (1) and **goat erythrocyte membrane** (2) on 10% polyacrylamide gels in 1% sodium dodecyl sulfate as visualized by coomassie blue stain (A) and carbocyanin stain (B). TD represents the position of tracking dye.

an almost similar pattern of polypeptides however, an additional band designated as gp 155 (mol wt. 155,000) by Inaba & Maede (1988) is visible between the bands 1 + 2 and band 3, which is a transmembrane glycoprotein.

Visualization of the human and goat erythrocyte membrane polypeptides with carbocyanin dye (Figure 14B, 1 and 2, respectively) resulted in the staining of sialoglycoproteins PAS I, II and III as blue bands. The most prominent of these PAS bands in human erythrocyte membrane electrophoretic profile has a molecular weight of 83,500 (Fairbanks *et al.*, 1971).

Different age group red blood cell membranes

Interaction of bilirubin (150 μM) with the erythrocyte membranes of different age groups shows maximum binding of bilirubin to old erythrocyte membranes (114.8 nmoles/mg phospholipid) and minimum with young erythrocyte membranes (59.6 nmoles/mg phospholipid). The difference in the amount of bound bilirubin was found to correlate negatively with sialic acid content and phospholipid content (as is evident from Table I), which is maximum (21 nmoles and 1.365 mg, respectively) in case of young erythrocytes and minimum (13 nmoles and 0.810 mg, respectively) in case of old erythrocyte membranes. Aging results in decrease of resistance to membrane permeability, which may account for the old cells sequestering more bilirubin. Moreover, reduction in the total sialic acid content per cell (Seaman *et al.*, 1977) connected with the loss in membrane surface area appears to determine minor repulsive forces (Danon & Marikovsky, 1988). The penetration of bilirubin into the cell membrane is

Table I

Comparison of bilirubin bound to different age groups of human erythrocyte membranes.

Age group	Phospholipid content (mg)	Sialic acid content (nmoles)	Bound bilirubin (nmoles)	Bound bilirubin/mg phospholipid (nmoles/mg phospholipid)
YOUNG	1.365±0.23	21.0±1.2	81.4±1.3	59.6±4.2
MIDDLE	1.145±0.12	18.0±2.2	86.4±2.2	75.5±4.2
OLD	0.810±0.23	13.0±2.3	93.0±1.1	114.8±5.2

Each reading is the mean of six independent observations.

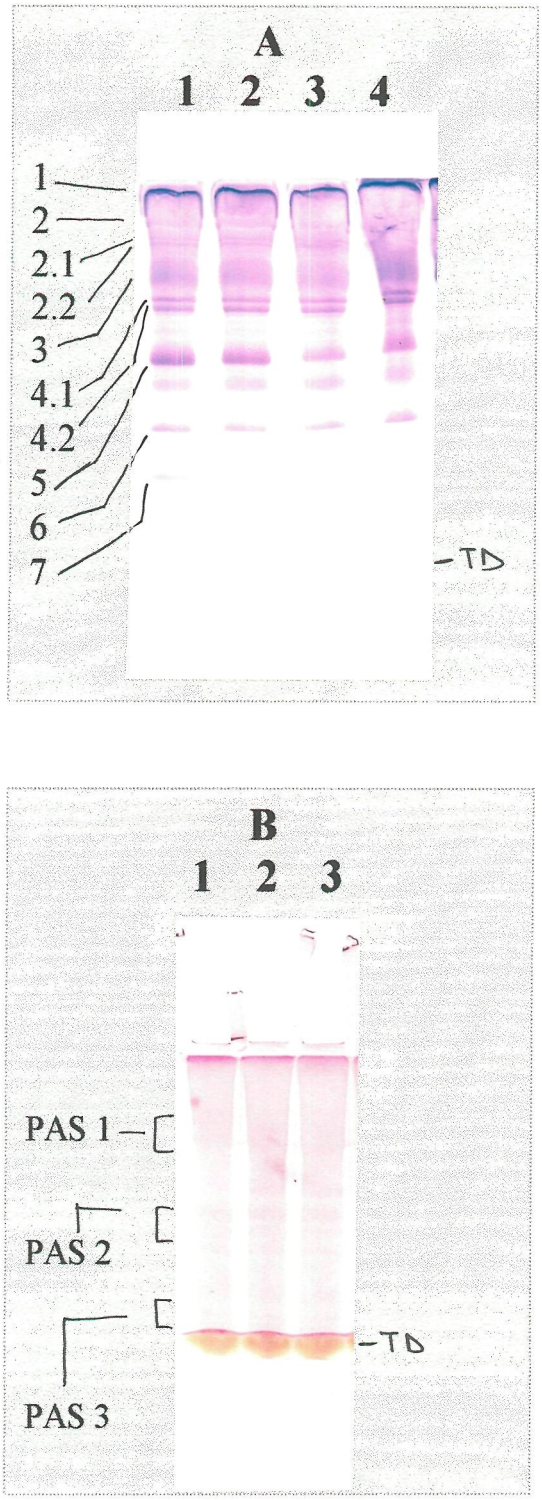


Figure 15. Electrophorogram (SDS-PAGE) of different aged group red cell membranes, unfractionated (1), young (2), middle (3) and old (4) as visualized by coomassie blue staining (A) and young (1), middle (2) and old (3) red blood cell membrane fractions as visualized by carbocyanin staining (B).

facilitated in senescent erythrocytes as compared to younger cells, due to diminished surface-charge density. The younger cells are more susceptible to crenation while older cells show increase in susceptibility to hemolysis at higher bilirubin concentration (Brites *et al.*, 1997).

The electrophoretic pattern as observed with coomassie stain showed no significant difference in the protein content and their specific polypeptide bands (shown in Figure 15 A, 1, 2, 3 and 4) however, carbocyanin staining (Figure 15 B) did show slight variation in their sialic acid content as evident by the reduction in the intensity of blue bands, signifying the position of glycopeptides. Cohen *et al.* (1976) also showed that sialic acid content decreases consistently with cell age. More of high molecular weight proteins in older cells due to peroxidation of lipids and lesser amounts of bands 1 and 2 proteins (spectrin) have been reported earlier (Balduni *et al.*, 1974) and phospholipid phosphorus has been reported to decrease (Westerman *et al.*, 1963) along with protein, which also decreases from top to bottom fraction i.e. increasing cell density (Rahman *et al.*, 1973) as seen from figure 15A (4). The biochemical profile of unfractionated cells is comparable with young and middle cell fraction (Cohen *et al.*, 1975). The young, middle and old erythrocyte membrane fractions (Figure 15A, 2, 3 and 4) have been compared with unfractionated cell membrane loaded into another well (Figure 15 A, 1).

The differences between the membrane glycoproteins of a very young and a very old erythrocyte must be much more evident than those shown in our results where significant differences in the binding of bilirubin to different aged group

cells is shown (the p-value of young versus middle, 0.0022; middle versus old, 0.0005 and young versus old 1.32×10^{-5}). Since the young and old 'ghosts' fractions, each correspond to about 10 % of the total erythrocyte population and it was not possible to isolate the cells just entered into the circulating blood from those that were going to be destroyed.

Interaction of bilirubin with erythrocyte membranes at different pH

At a given pH, increase in the B/A from 0.5 to 3.0 led to an increase in the membrane-bound bilirubin (Figure 16). This increase was smaller up to a B/A of 1.5:1 but became more significant ($P<0.05$) at high B/As at all the pH values used in this study. Nearly two-fold ($P<0.05$) increase in membrane-bound bilirubin was noticed on increasing the B/A from 1.0 to 2.0. At all the five different pH values, i.e., 7.0, 7.2, 7.4, 7.6 and 7.8, the patterns of bilirubin binding to membranes were qualitatively similar. However, the amount of membrane-bound bilirubin at any B/A in the range of 0.5 to 3.0 was different at different pH values, being negatively correlated with pH of the medium. In other words, lowering the pH from 7.8 to 7.0 resulted in increased binding of bilirubin to erythrocyte membranes at all the B/As tested even at B/As below 1:1. In an earlier study, cellular binding of bilirubin has also been shown to vary with pH in the same fashion (Bratlid, 1972b).

At a given constant B/A (i.e. 2:1), the binding of bilirubin to erythrocyte membranes increased linearly with increase in bilirubin concentration at all the pH values used (as shown in Figure 17). Similar results were found with other

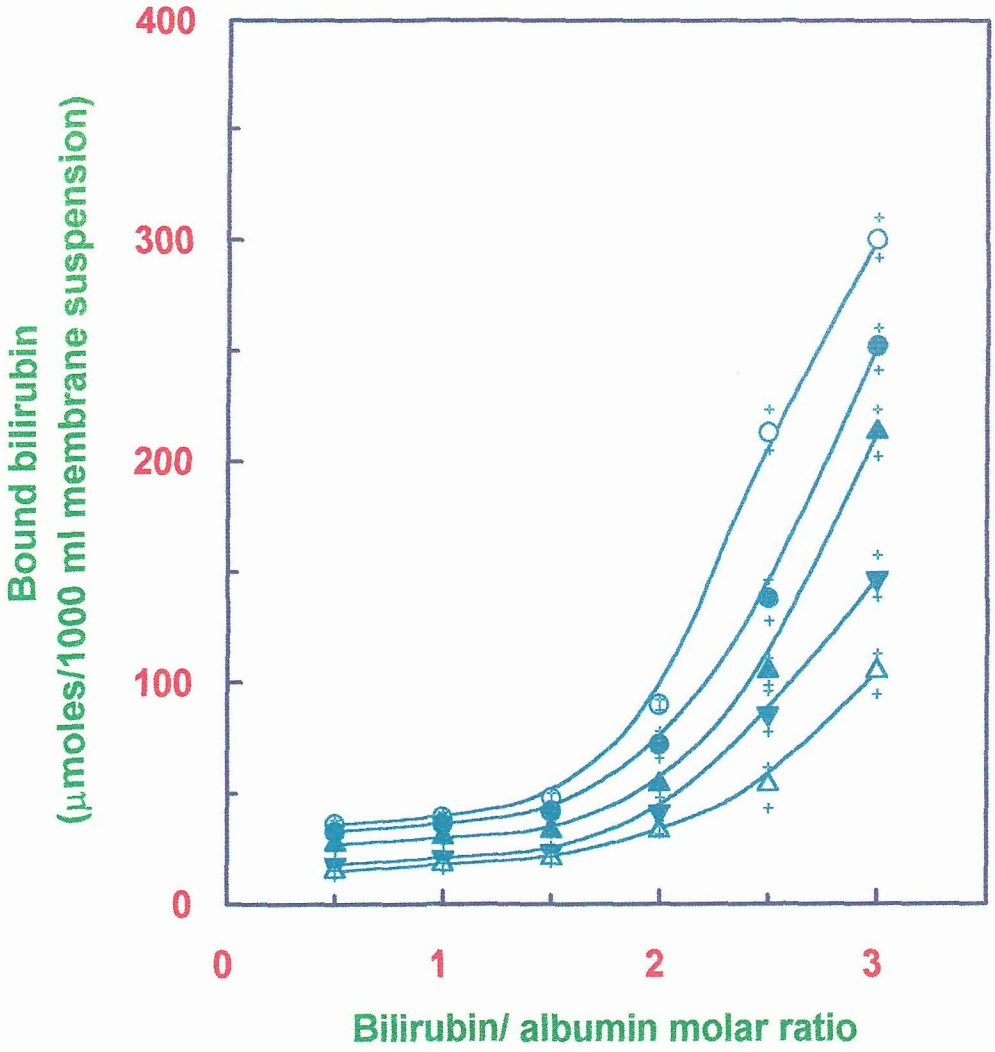


Figure 16. Binding of bilirubin to human erythrocyte membranes at different B/As and at different pH values [pH 7.0 (○), pH 7.2 (●), pH 7.4 (▲), pH 7.6 (▼) and pH 7.8 (△)]. Each point is the mean ± SEM of three independent observations.

B/As. Linear increase in bound bilirubin with the increase in bilirubin concentration in incubate at a constant B/A and pH was also observed earlier with human erythrocytes (Hayer *et al.,* 1989; Tayyab & Ali, 1997). Increase in membrane-bound bilirubin per unit increase in bilirubin concentration in incubate was calculated from the slope values of the straight-line plots obtained at different pH values and at different B/As. This slope value was found to be different for different pH values and different B/As, being highest at pH 7.0 (Figure 18). However, at B/As 2.5 and 3.0, the value of slope became constant between pH 7.4 and 7.0. In other words, the increase in membrane bound bilirubin per unit increase in bilirubin concentration in incubate was similar between pH values 7.4 and 7.0 at B/As 2.5 and 3.0. As the binding of bilirubin to erythrocytes and erythrocyte membranes follows a Michaelian saturation curve (Tayyab & Ali, 1995; Hayer *et al.,* 1989), the slope value calculated from curves shown in figure 17 will depend on the range of free bilirubin concentration available in the incubate in such a way that with the increase in the range of free bilirubin concentration, the change in the slope value will be minimum. For example, at pH 7.4, the difference in slope value obtained was maximum between B/As 2.0 and 1.5 followed by between 2.5 and 2.0 whereas the value was minimum between B/As 3.0 and 2.5 (see figure 18). Since the available range of free bilirubin concentration at B/A 1.5 is minimum whereas it is maximum at B/A 3.0, these results are in accordance with the above explanation. One possible factor for the higher binding observed at pH 7.0 compared to pH 7.4 or higher may be the large increase in free bilirubin concentration in the

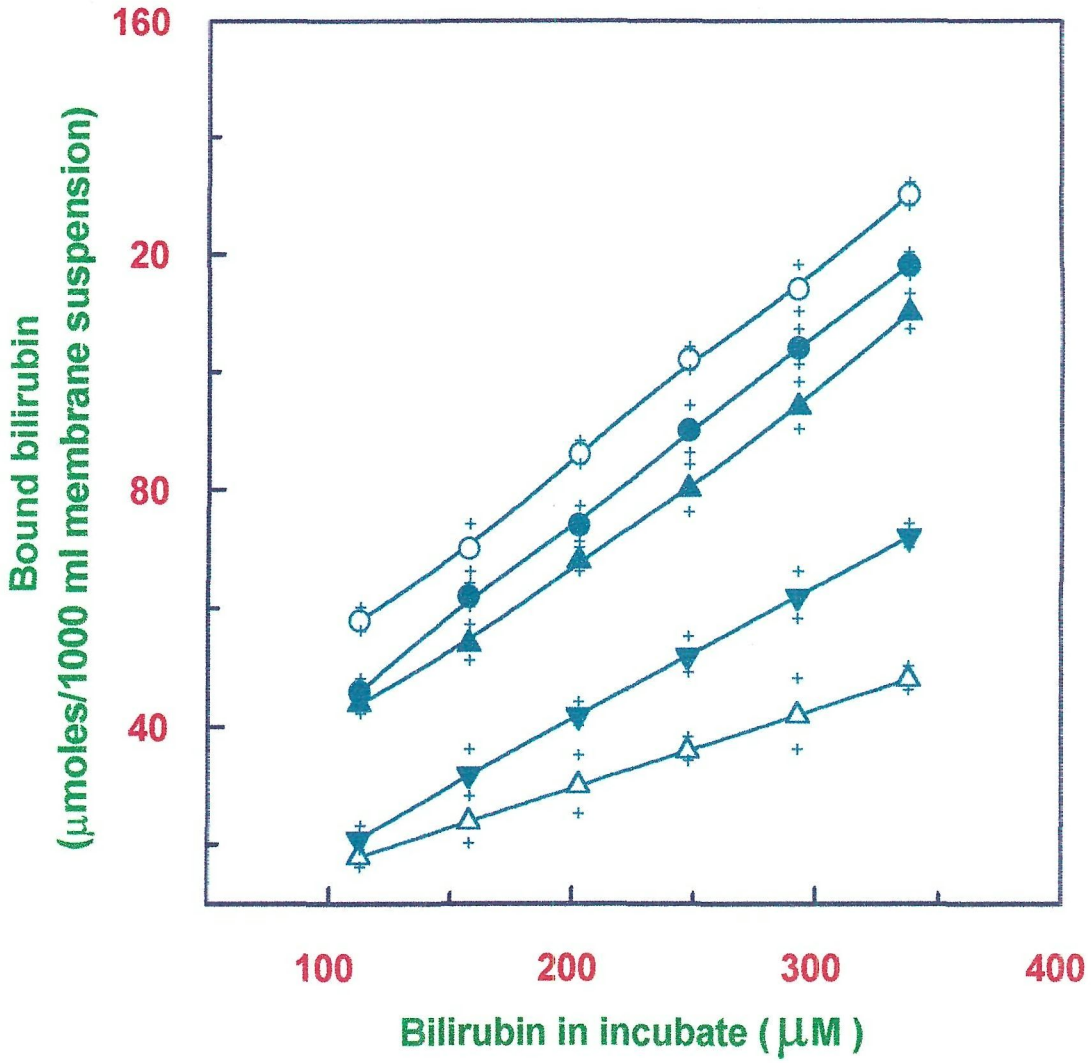


Figure 17. Binding of bilirubin to human erythrocyte membranes at constant B/As but increasing bilirubin concentrations and at different pH values [pH 7.0 (○), pH 7.2 (●), pH 7.4 (▲), pH 7.6 (▼) and pH 7.8 (△)]. Each point is the mean ± SEM of three independent observations.

incubate at pH 7.0 compared to pH 7.4 due to decreased albumin binding as reduced binding of bilirubin to albumin has been shown earlier by different workers after lowering the pH from 7.4 to 6.5 (Odell *et al.,* 1969). Another factor may be the increased susceptibility of erythrocyte membranes towards bilirubin at lower pH. This is because decrease in pH may convert bilirubin dianion (B^{2-}) to monoanion (BH^-), which seems to be responsible for the increased binding of bilirubin to membranes at lower pH (Vazquez *et al.,* 1988). It may be noted that out of the three species of bilirubin i.e., bilirubin dianion (BH^{2-}), bilirubin monoanion (BH^-) and bilirubin acid (BH_2), erythrocyte membranes are capable of binding both BH_2 and BH^- (Vazquez *et al.,* 1988; Brites *et al.,* 1997). Keeping in view all the above points, consistency in the slope value observed in the pH range 7.4-7.0 at higher B/As (2.5 and 3.0) can be ascribed to a higher free bilirubin concentration in the incubate. Since membrane binding of bilirubin is significantly increased with decreasing pH even at lower B/A i.e. 1.5, the results shown here are of clinical significance as B/A of as high as 1.37 has been reported in jaundiced neonates (Cashore & Oh, 1982) and also in the presence of various bilirubin displacing ligands including drugs, the B/A may reach a higher value which may be fatal under acidosis conditions.

Interaction of bilirubin with erythrocyte membranes at different temperatures

Incubation of erythrocyte membranes with bilirubin at different temperatures ranging from 7°C to 60°C showed that at any given B/A above 1:1, the amount

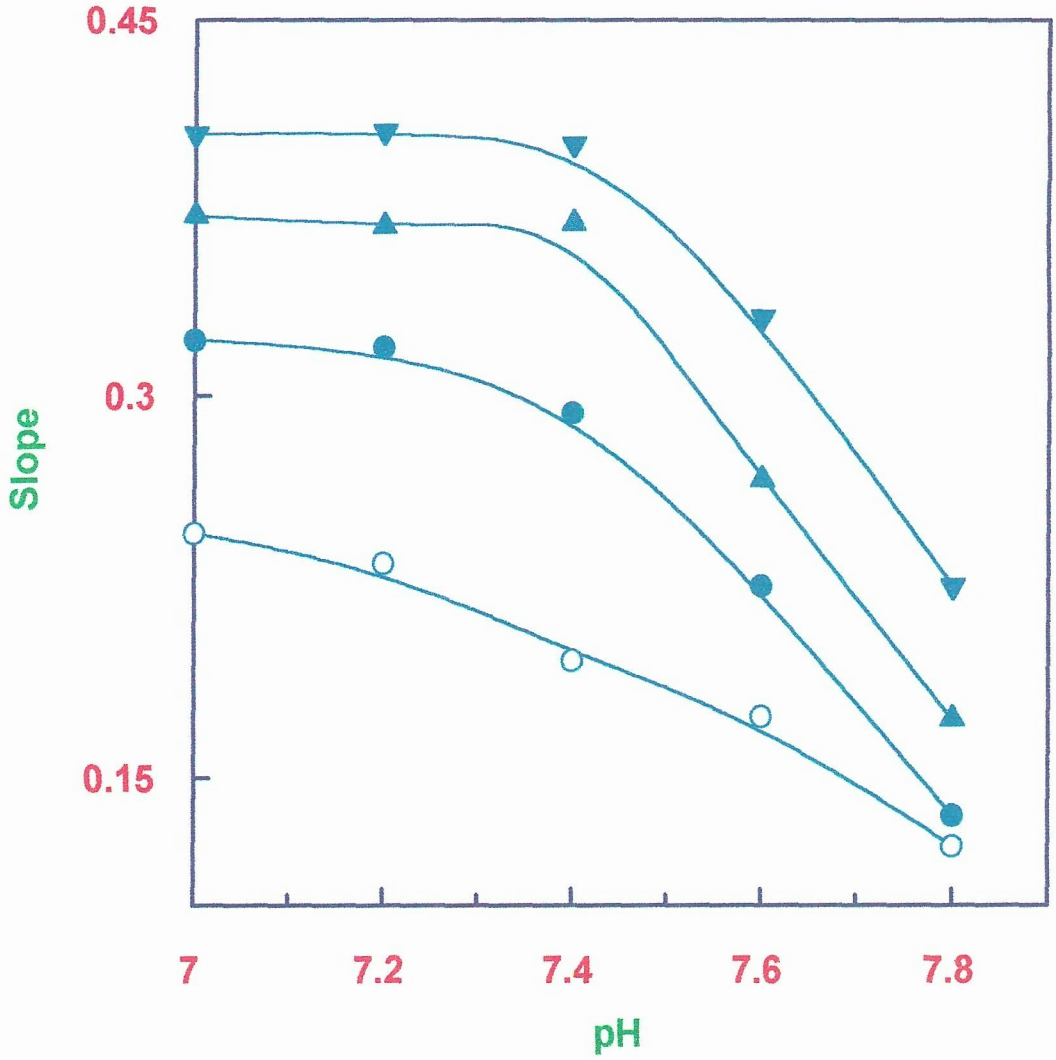


Figure 18. Plots of the slope values of the straight line plots obtained in figure 17 versus pH [B/A 1.5 (○), B/A 2.0 (●), B/A 2.5 (▲), B/A 3.0 (▼)].

of membrane-bound bilirubin was greatly influenced by the temperature of the incubation medium as shown in figure 19. Within the temperature range of 27°C to 37°C, the amount of membrane-bound bilirubin at any given B/A above 1:1 was found to be minimum. Increase in temperature on either side led to an increase in bilirubin binding to erythrocyte membranes at all B/As. Sato & Kashiwamata (1983) also reported minimum binding of bilirubin to erythrocyte membranes at 37°C using a B/A of 2.0. A comparison of bilirubin binding patterns of human erythrocyte membranes at different temperatures and B/As shows that at each temperature, the binding was higher at higher B/As. This unusual behavior of bilirubin binding to erythrocyte membranes at different temperatures may be either due to the effect of temperature on the binding of bilirubin to albumin or due to the change in the physical state of bilirubin binding sites of the erythrocyte membranes. The lipid bilayer of human erythrocyte membrane exists in gel state below 18.5°C while above 40°C it mainly exists in liquid crystalline state (Barenholz & Thompson, 1980). Cestaro *et al.* (1983) have reported that in the gel state both disordered phospholipid bilayers and protrusion of apolar regions of membrane proteins highly increased the hydrophobicity of outer sides of the membranes which greatly potentate the binding of bilirubin to the membranes. On the other hand, above 40°C, the membranes are in liquid-crystalline state and the lipids are randomly arranged (Vigh *et al.,* 1998) and both the surface area per molecule and bilayer spacing are increased due to the hydration of phospholipids (Barenholz & Thompson, 1980). Under such conditions, bilirubin can freely penetrate the internal apolar core of

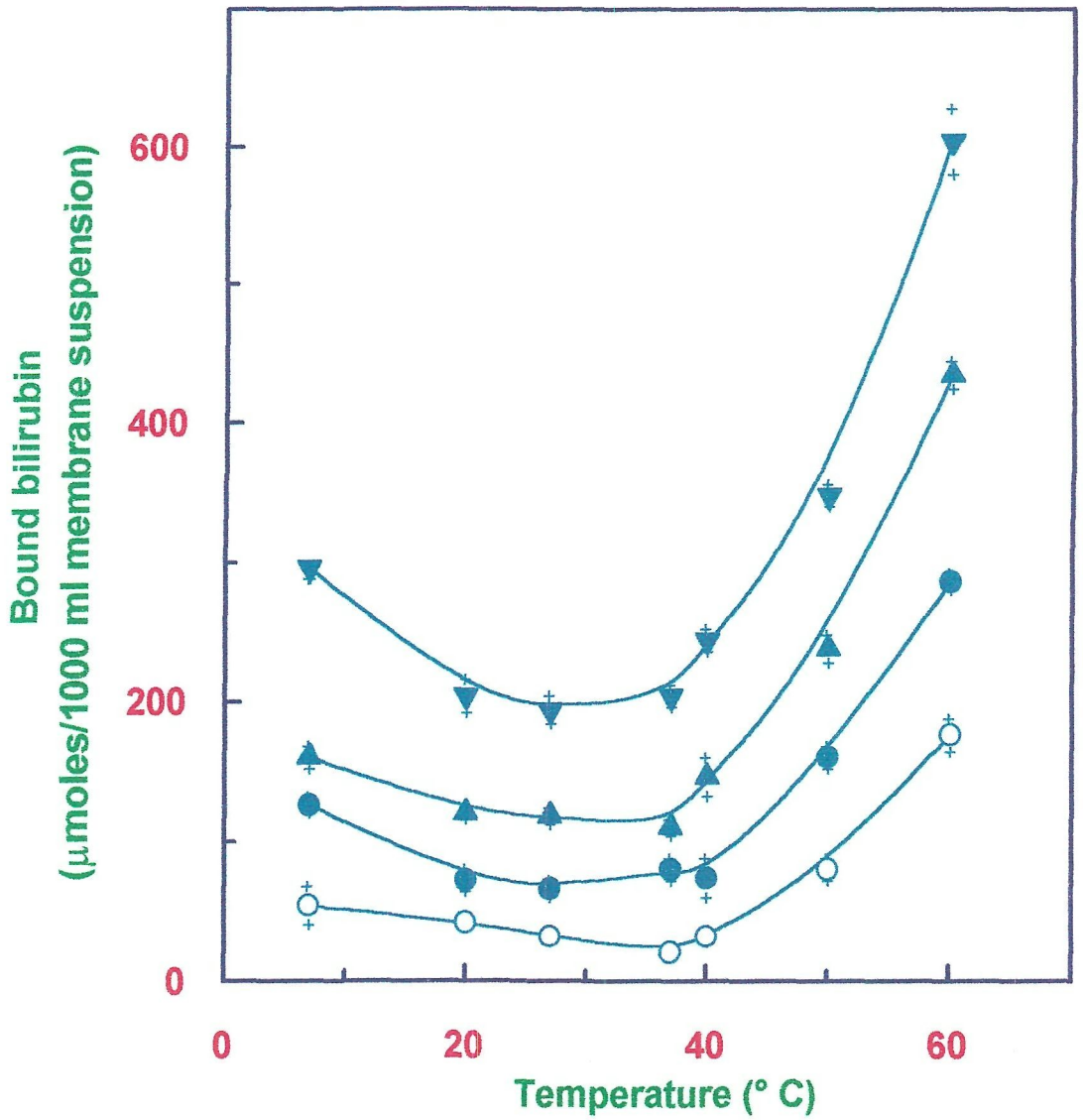


Figure 19. Binding of bilirubin to human erythrocyte membranes at a given B/A and pH but at different temperatures [B/A 1.5 (○), B/A 2.0 (●), B/A 2.5 (▲), B/A 3.0 (▼)]. Each point is the mean ± SEM of three independent observations.

the membrane bilayer from both surfaces (Hayward *et al.,* 1986). However, within the temperature range of 20-40°C, the arrangement of lipid in the bilayer of the membrane is not random, i.e. most of the choline phospholipids are confined to the outer while negatively charged phospholipids to the inner surface. Under such condition, it is likely that the interaction of bilirubin with the outer surface is more favorable than that of the inner surface, which may account for the low binding of bilirubin within the temperature range of 20-40°C. In addition to this, role of temperature in decreasing the bilirubin-albumin interaction thereby increasing the free bilirubin concentration for binding to the membranes cannot be ruled out.

Differential accessibility of bilirubin to erythrocyte membrane vesicles bearing different structural features

Figure 20 shows the results of binding of bilirubin to different human erythrocyte membrane preparations at a fixed bilirubin/ albumin molar ratio (2:1) but at increasing concentrations of bilirubin (120-380 μM). As is evident from the figure, increase in the concentration of bilirubin in the incubate mixture led to an increase in the membrane-bound bilirubin in all types of membrane preparations. Although, the pattern of increase was similar in all types of membrane preparations, they showed quantitative differences in the amount of bound bilirubin. Maximum binding of bilirubin was found in unsealed membranes followed by heterogeneous and sealed membrane vesicles whereas minimum binding of bilirubin was observed in case of inside out membrane vesicles.

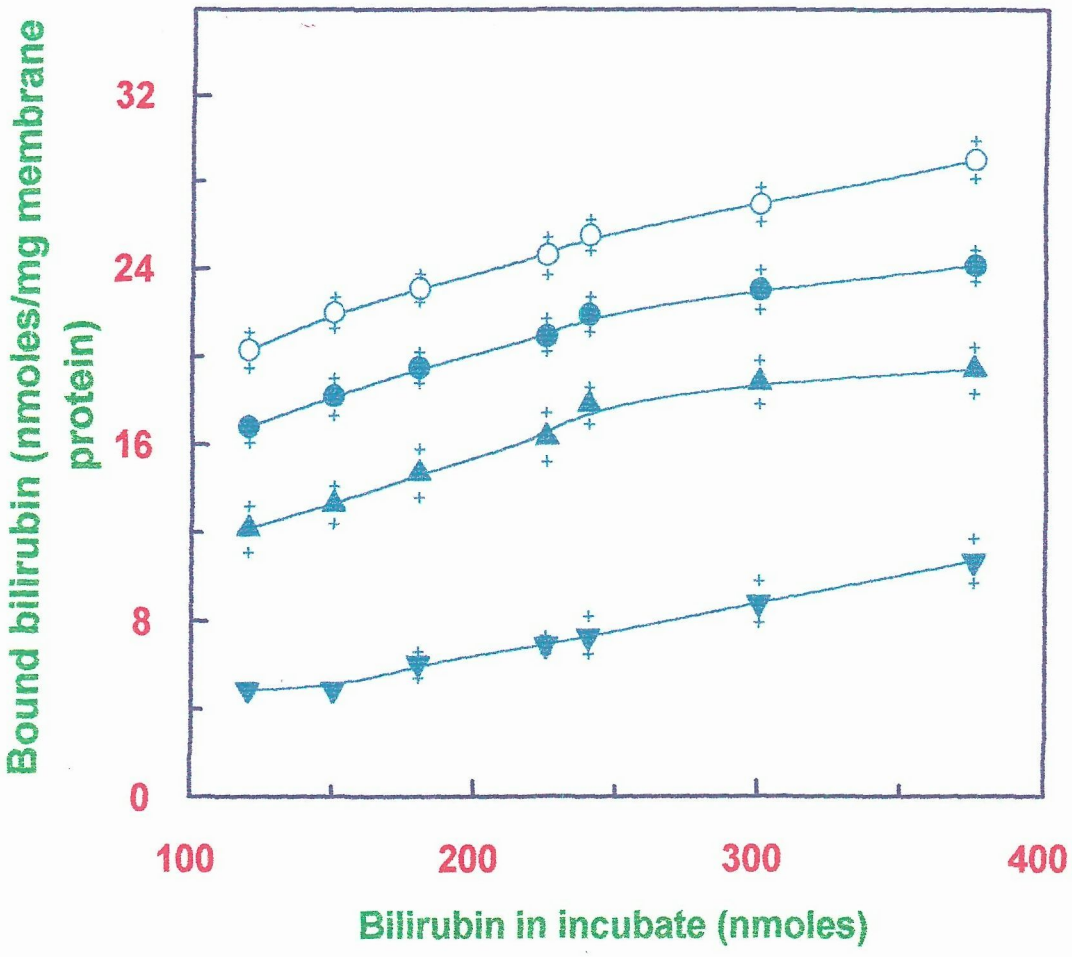


Figure 20. Binding of bilirubin to the different types of human erythrocyte membrane vesicles: unsealed (○); heterogenous (●); sealed (▲) and inside out (▼). Each value is the mean ± S.D. for three separate determinations.

Statistically, the difference in the amount of membrane-bound bilirubin between unsealed and heterogeneous, heterogeneous and sealed as well as sealed and inside out vesicles was found highly significant ($P<0.05$). The analysis of bilirubin binding data to inside out and sealed vesicles suggest that the inner surface of the membranes are able to bind some amount of bilirubin indicating the presence of few bilirubin binding sites on the inner surface of the membranes. However, the bilirubin binding sites presence on the outer surface of membranes seem to be comparatively large in number than that of inner surface. This assumption is further justified by the observations that the binding of bilirubin to unsealed membranes in which both the inner and outer layer of the membranes is available for bilirubin binding, bind more bilirubin than the sealed membranes in which only the outer layer is available for bilirubin binding. Further, comparison of bilirubin binding to heterogeneous membranes (i.e., mixed population of unsealed and sealed membranes) with that of unsealed and sealed shows that the binding is lower than that of unsealed membranes but higher than that of sealed membranes suggesting that the assumption is in good agreement with the observed data. This order of bilirubin binding to different types of membrane preparations was found to correlate with the percentage accessibility of neuraminidase. The percentage accessibility of neuraminidase was in the order of 89.7%, 83.3%, 78.9% and 42.1% for unsealed, heterogeneous, sealed and inside out membranes respectively. In all these membrane preparations the total sialic acid as well as the total phospholipid content was found more or less similar, suggesting that the difference in the binding of bilirubin to these erythrocyte

membrane preparations may be due to different structural features of membranes generated under different conditions. Since the released of sialic acid when treated with neuraminidase depend upon the availability of outer layer of the membranes as sialic acid is mainly located at the outer surface of bilayer, the binding of bilirubin to the different types of membranes species also seem to be depend upon the availability of outer layer of the membranes. In other words, it seems that greater the exposure of inner layer of membranes, more is the reduction in the binding of bilirubin to the membranes. This seems to be highly reasonable as the negatively charged bilirubin mono anion is reported to involved in the interaction of the membranes, therefore the exposures of high negative charge density on the inner surface of membranes due to presence of phosphatidic acid and phosphatidylserine will repel the binding of bilirubin to the inner surfaces. The binding of bilirubin to the outer surface will be more favorable as the presence of sialic acid on the outer surfaces of membranes have negligible impact on the binding of bilirubin to the erythrocyte membranes (Sato *et al.,* 1987).

Similar bilirubin binding pattern have also been found with the different types of membranes prepared from goat erythrocytes and is shown in figure 21. However, the amount of bound bilirubin was much less compared to that of human erythrocytes. Further, significant differences were observed only between the unsealed and inside out (P= 0.002), heterogeneous and inside out (P= 0.01), sealed and inside out membrane vesicles (P= 0.02) whereas marginally significant differences was found between unsealed and sealed membrane

vesicles (P= 0.07). Estimation of percent accessibility of neuraminic acid also revealed that the percent sialic acid released in unsealed and heterogeneous membranes are nearly similar but marginally different from sealed and inside-out membrane vesicles. The different in bound bilirubin among different types of membranes preparations therefore was more or less positively correlated with the percent accessibility of neuraminidase to these membranes. As these experiments involving human and goat erythrocyte membranes were performed by taking similar volumes of membrane suspensions containing same amount of membrane protein, the differences in the binding of bilirubin to different types of membrane preparations between human and goat seem to be due to the difference in phospholipid composition of these membranes. As goat erythrocyte membranes have high content of sphingomyelin and little phosphatidylcholine compared to human erythrocyte membranes having high content of phosphatidylcholine (Barenholz & Thompson, 1980), this can account for the difference in the binding of bilirubin to these two types of membranes (also from table II). Earlier we have shown that maximum amount of membrane-bound bilirubin is hydrophobically inserted in the lipid bilayer which cannot be removed by the addition of albumin (Tayyab & Ali, 1999). Recalling that the interaction of bilirubin with sphingomyelin mainly involves hydrogen bonding (Zucker *et al.*, 1992) and dual nature of bilirubin binding to biomembranes in the form of dianion which binds weakly to the polar heads of the phospholipids and bilirubin acid binding strongly and is hydrophobically inserted into the apolar region of the bilayer (Cestaro *et al.* 1983), it appears that phosphatidylcholine rich human

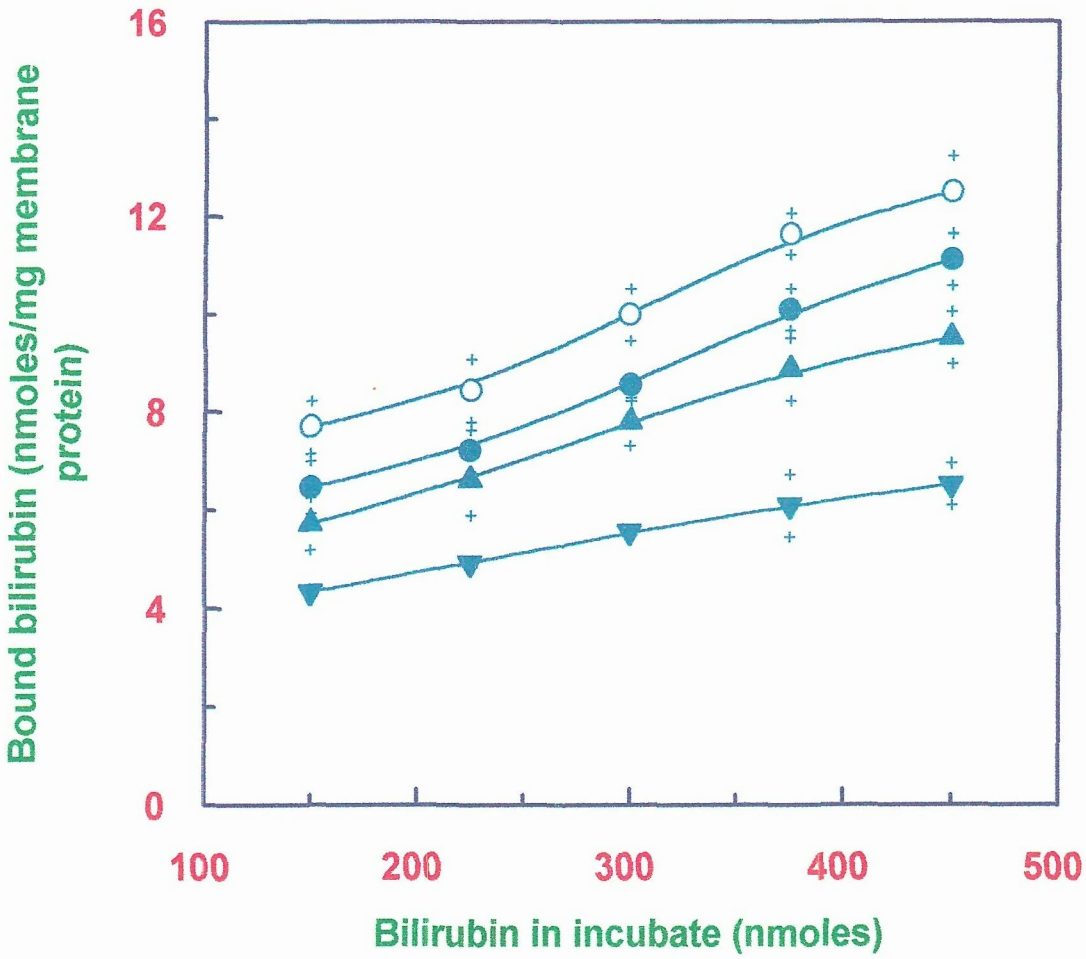


Figure 21. Binding of bilirubin to the different types of goat erythrocyte membrane vesicles: unsealed (○); heterogeneous (●); sealed (▲) and inside out (▼). Each value is the mean ± S.D. for three separate determinations.

erythrocyte membranes are better candidates for bilirubin binding than goat erythrocyte membranes. This is supported by an earlier finding that the amount of hydrophobically inserted bilirubin at a given time of incubation was found much lower in sphingomyelin containing membranes compared to phosphotidylcholine containing membranes (Eriksen *et al.,* 1987). Further, higher rigidity of goat erythrocyte membranes due to high sphingomyelin content (Barenholz & Thompson, 1980) may act as a barrier in the hydrophobic insertion of bilirubin in these membranes. This is further supported by the fact that the uptake of amphiphathic molecules to human erythrocytes is much higher that than of goat erythrocytes (Farooqui *et al.,* 1987). In spite of the differences in the amount of bilirubin binding to erythrocyte membranes of goat and human, the similar differences in the binding of bilirubin to different erythrocyte membrane vesicles of each species suggest either bilirubin binding sites of the erythrocytes membranes are mainly concentrated on the outer layer of membranes as suggested by Vazquez *et al* (1988) or both sides of the membranes are capable of binding bilirubin but the degree of bilirubin interaction were seems to be determined by the phospholipid compositions of each layer as suggested by (Zucker *et al.,* 1992; 1994). In other words it seems that the outer layer of erythrocyte membranes having high content of PC and Sph were more susceptible to bilirubin compared to the inner layer of membranes containing high concentration of acidic phospholipids.

Table II.

Choline phospholipids and sialic acid contents of human and goat erythrocyte membranes

	Human	Goat
Total phospholipid (ng/mg protein)	348.2±7.9	319.0±13.5
Phosphatidylcoline* (ng/mg protein)	130.6±2.8	11.3±0.5
Sphingomyelin* (ng/mg protein)	86.0±2.0	146.4±6.2
Sialic acid** (nmoles/mg protein)	44.0±1.7	25.9±0.9

*Both phosphatidylcholine and sphingomyelin of human and goat erythrocytes membranes are calculated from the total phospholipids using known percentage of these phospholipids contents (Barenholz & Thomson, 1980).

** Sialic acid (nmoles/mg protein) is the total sialic acid release from (0.2% v/v) Triton X-100 treated membranes after incubation with 0.1mg/ml neuraminidase for 30 min at 25°C.

Effect of phospholipases, neuraminidase, trypsin and $CaCl_2$

The effect of phospholipase C (*C. welchii)* treatment of human erythrocyte membranes can be seen from figure 22 where the amount of membrane Pi released is plotted against time of incubation of the membranes with the enzyme. A significant increase in the amount of released Pi was observed up to 40 min of incubation, which became constant thereafter. Using the value of total membrane Pi content (Table IV), the enzyme treatment resulted in the release of about 62.3% of the membrane Pi. Similar enzymatic treatment of bilirubin-bound membranes (previously incubated with 450 nmoles of bilirubin at B/A of 2.0 and washed subsequently) showed a significant reduction in the amount of Pi released for the initial time periods up to 30 min. However, increase in the time of incubation up to 60 min was found to produce the similar release of membrane Pi as found with unbound membranes. Recalling that phospholipase C treatment of erythrocyte membranes results in the release of polar head groups from phosphatidylcholine (PC) and sphingomyelin (Sph) from the outer layer of erythrocyte membranes, the lesser release of membrane Pi in the early periods observed with bilirubin-bound membranes can be ascribed to the protective effect of bound bilirubin. It appears that removal of a few polar head groups of phospholipids from the outer layer facilitates the release of more Pi over a period of time, which seems to be hindered in the initial phase by the membrane-bound bilirubin. Extensive enzymatic treatment of the membranes led to enhanced packing of the ghosts, however, the cellular morphology is retained as suggested by Lenard & Singer (1968).

Table III

Chemical composition of human erythrocyte membranes (equivalent to 1.0 ml of 50 % hematocrit value)

Component	Amount
Protein (mg)	3.8±0.2
Total phosphorus (Pi, μg)	111.7±4.2
Organic phosphorus* (μg)	53.0±1.7
Phospholipid (mg)	1.3±1.7
Sialic acid (nmoles)	119.5±8.7

*Pi as determined in isopropanol-chloroform extracted phospholipids.

Each value is the mean ± S.D. of twelve observations obtained with six different membrane preparations.

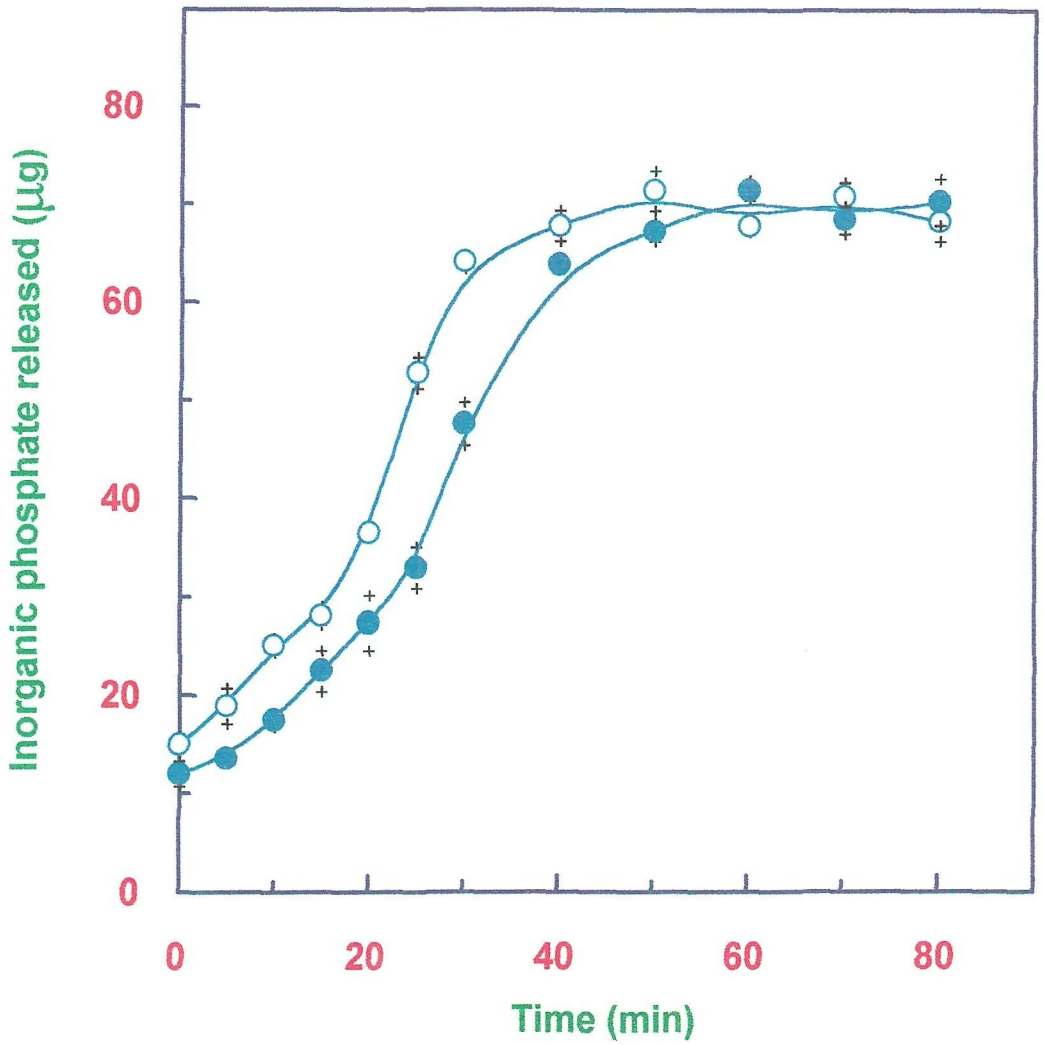


Figure 22. Time course of phospholipase C (*C. welchii*) treatment of human erythrocyte membranes as monitored by Pi release. Open circles show the amount of Pi released upon enzyme treatment from normal membranes whereas closed circles represent Pi released from bilirubin-bound membranes. Each point is the mean of two independent experiments.

Incubation of untreated as well as phospholipase C-treated human erythrocyte membranes with constant amount of bilirubin (150 nmoles at B/A of 2.0) led to an increase in the membrane-bound bilirubin. As much as 121% increase in membrane-bound bilirubin was noticed with the modified membranes treated with enzyme for 40 min (Figure 23). This increase in the membrane-bound bilirubin upon phospholipase C treatment correlated very well with the release of membrane Pi, which suggests that polar head groups of phospholipids play inhibitory role in the bilirubin-binding phenomenon. Even a very short (5 min) treatment of the membranes with phospholipase C resulted in 18% increase in membrane-bound bilirubin. These results were similar to the results observed by Sato and his group (1987). As phospholipase C treatment of bilirubin-bound membranes failed to release the bound bilirubin from these membranes, which otherwise resulted in the release of Pi, it appears that bilirubin binding to the membranes involves hydrophobic interactions. This is supported by an earlier finding, in which it has been suggested that bilirubin is hydrophobically inserted into the apolar region of the bilayer (Zucker *et al.*, 1994).

Degradation of phospholipids of human erythrocyte membranes by phospholipase C as analysed by thin-layer chromatography (Figure 24 and table IV) shows about 70% degradation of total phospholipids, which comprised of total degradation of Sph, 86% degradation of PC and 28% of phosphatidylethanolamine (PE) after 20 min of enzyme treatment. This suggests that exposure of diacylglycerols or ceramides potentiated a large increase in the membrane-bound bilirubin.

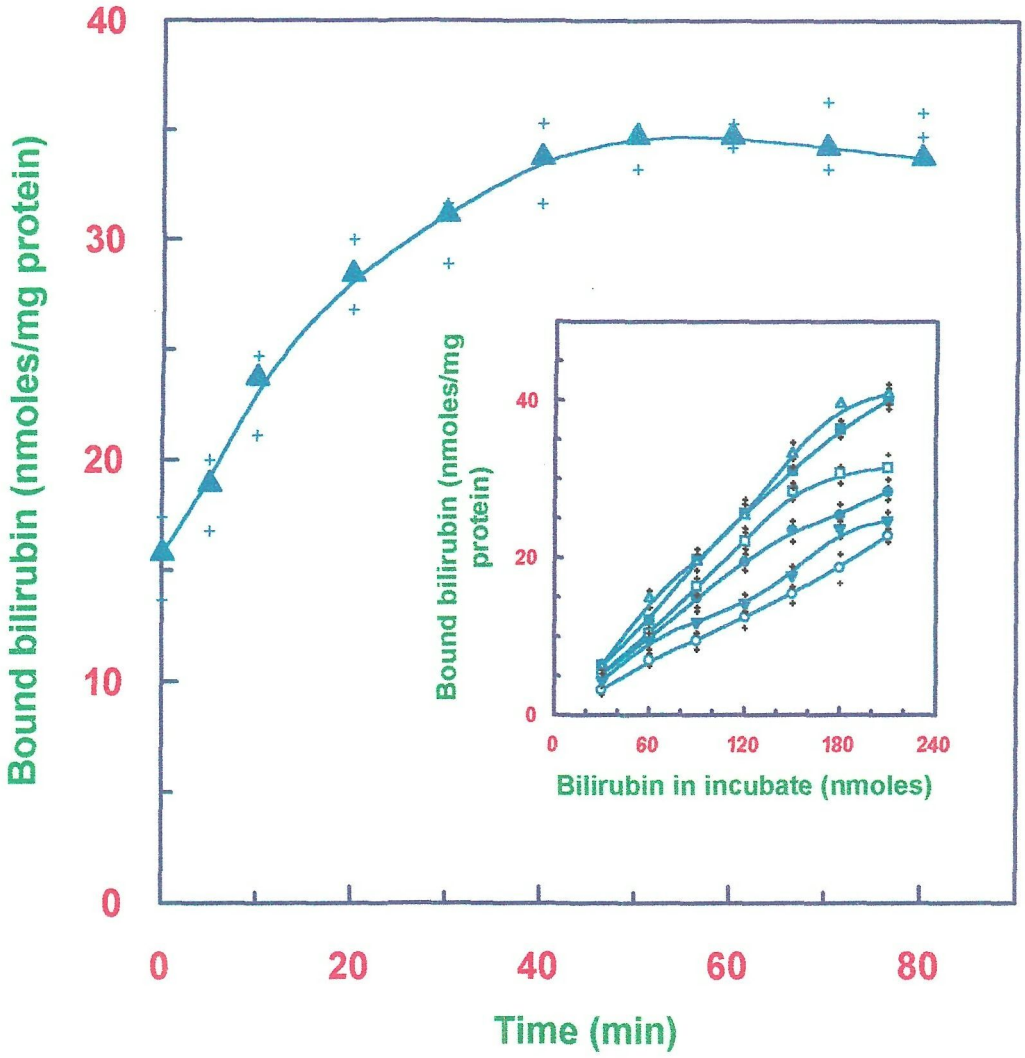


Figure 23. Effect of phospholipase C (*C.welchii*) on the binding of bilirubin. Amount of bilirubin bound to phospholipase C-treated membranes is shown by closed triangles. Each point is the mean of two independent experiments.

Inset shows the amount of bilirubin bound with increasing bilirubin concentration in incubate mixture for different time periods of phospholipase C treatment, 0 min (°); 5 min (▾); 10 min (•); 20 min (▫); 40 min (▪) and 60 min (▵).

Phospholipase D treatment of erythrocyte membranes for varying time periods was monitored by the estimation of choline released (Figure 25), as the enzyme is known to remove polar head groups from PC, PE and phosphatidylserine (PS) to produce phosphatidic acid. The enzymatic digestion of membranes for 60 min resulted in the release of 47.6 μg of choline/mg of protein, which corresponded to the degradation of about 45% of PC. Presence of bilirubin at the membrane surface, as observed with erythrocyte membranes pre-treated with 450 nmoles of bilirubin at B/A of 2.0, significantly inhibited the release of choline as 38.5% degradation of PC was noticed after phospholipase D treatment for 60 min. The lower extent of choline released from bilirubin-bound membranes observed at prolonged enzyme treatment can be attributed to the inaccessibility of enzyme cleavage sites due to bound bilirubin. Lack of difference in the amount of choline released from both normal and bilirubin-bound erythrocyte membranes at shorter incubation with the enzyme and the significant reduction in the amount of choline released from bilirubin-bound membranes compared to normal membranes at prolonged incubation of enzyme with these membranes suggests that release of few choline molecules from PC results in the clustering of bilirubin molecules around PC molecules, thereby, making some of the cleavage sites inaccessible to the enzyme.

Bilirubin binding results obtained with phospholipase D-treated membranes at B/A of 2.0 (with 225 nmoles of bilirubin) showed 23% increase in the bound bilirubin upon prolonged enzyme treatment (60 min) compared to untreated membranes (Figure 26). Although these results suggested the inhibitory role of

Table IV

Quantitative analysis of phospholipid degradation of human erythrocyte membrane by phospholipase C (*C.welchii*) under different time periods

Time (min)	% Phospholipid recovered after treatment			
	Total	PE	PC	Sph
0	88.1±7.8	23.9±1.8	30.1±2.8	23.8±1.2
20	26.4±6.0	17.0±2.8	4.3±1.8	-
40	25.7±4.6	15.3±2.2	4.7±1.1	-
60	26.5±4.6	17.2±2.1	4.2±1.6	-

Each value represents the mean ± S.D. of six observations obtained with three different experiments.

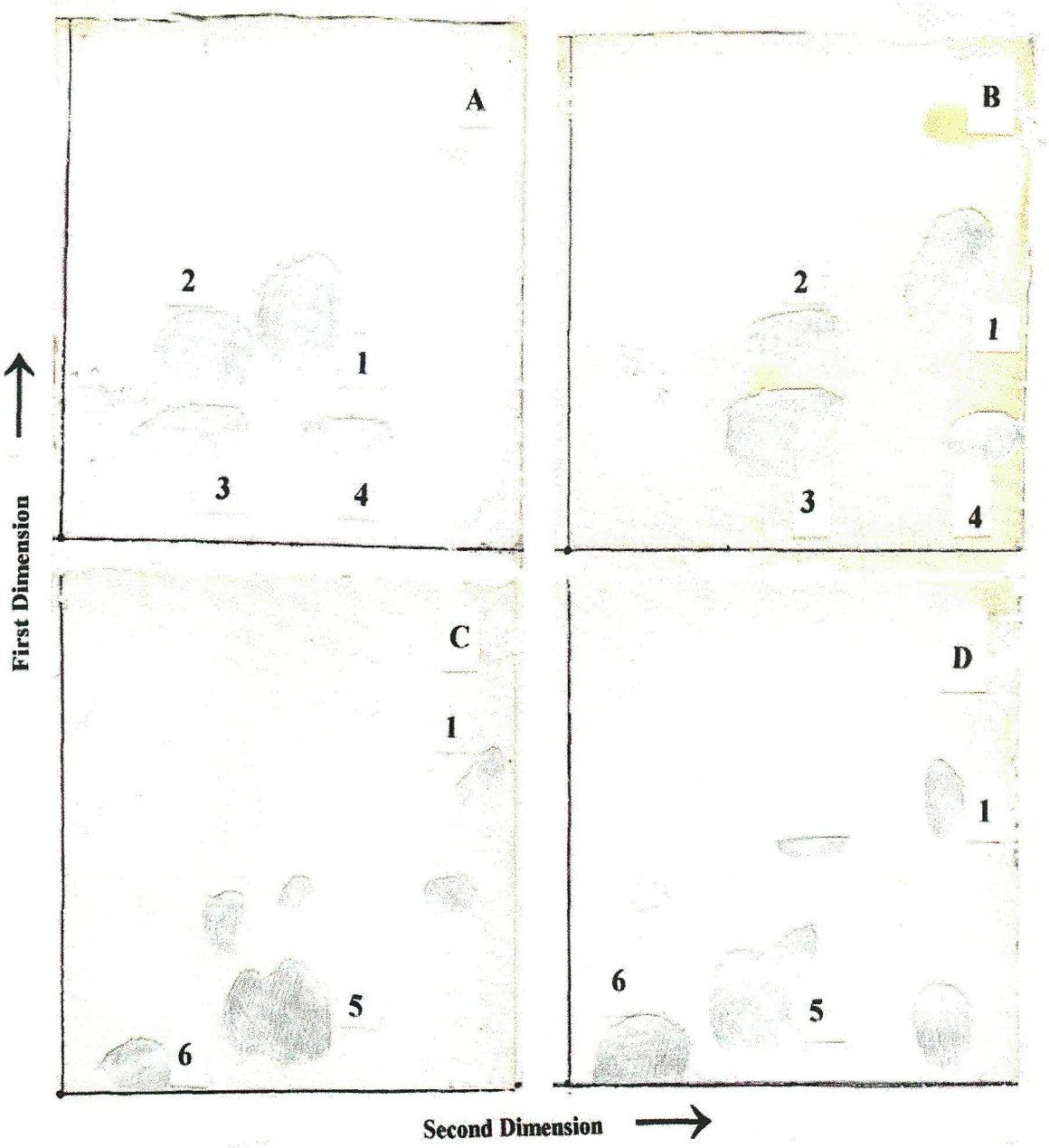


Figure 24. Two-dimensional chromatogram of phospholipids from human erythrocyte membrane, visualized by iodine vapors before phospholipase C (*C.welchii*) treatment (A) and after phospholipase C (*C.welchii*) treatment for 20 minutes (B), 40 min (C) and 60 minutes (D).

The solvents are chloroform-methanol-28% ammonia (65:35:5, v/v) for the first dimension solvent. The numbers represent individual phospholipids,

(1) Phosphatidylethanolamine (PE), (2) Phosphatidylcholine (PC), (3) Sphingomyelin (Sph), (4) Phosphatidylserine (PS), (5) Lysophosphatidylethanolamine (LPE) and (6) Lysohosphatidylserine (LPS).

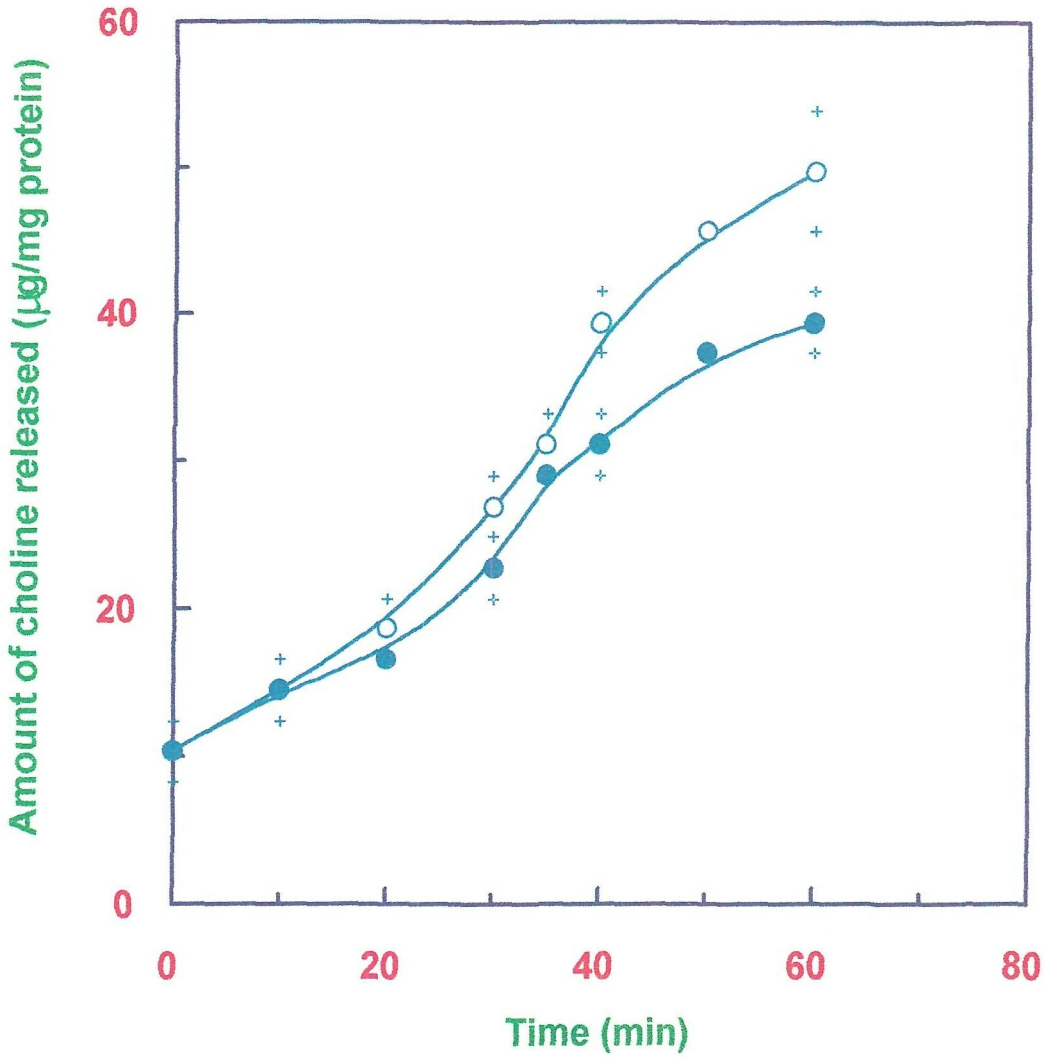


Figure 25. Time course of phospholipase D (Cabbage) treatment of *human erythrocyte membranes as monitored by choline release.* Open circles (○) show the amount of choline released upon enzyme treatment from normal membranes whereas closed circles (●) represent choline released from bilirubin-bound membranes. Each point is the mean of two independent experiments.

polar head groups in bilirubin-binding to the membranes to some extent, bilirubin-binding results obtained with phospholipase C-treated membranes were found to be more convincing as 121% increase in bilirubin-binding was observed upon phospholipase C treatment against 23% increase in phospholipase D-treated membranes. From these results it appears that the presence of negative charge contributed by Pi restricts the bilirubin entry inside the hydrophobic milieu of membrane to a greater extent. In view of the stimulation of endogenous phospholipases (C and D) by receptor tyrosine kinases (Schmidt *et al.*, 2000), growth factors (Shen *et al.*, 2001), tumour necrosis factor α (Plo *et al.*, 2000) etc., it is likely that membranes of various cells may be acted upon by these phospholipases and thus become more prone towards bilirubin toxicity. Therefore, activities of endogenous phospholipases should be checked to prevent bilirubin toxicity under jaundiced conditions.

Incubation of human erythrocyte membranes with neuraminidase for varying time periods resulted in the release of sialic acid. Time course of neuraminidase-treatment of erythrocyte membranes is shown in figure 27, which suggests that greater incubation of membranes with the enzyme led to a greater release of sialic acid that became constant after 60 min. A value of 84.1 nmoles of sialic acid / mg of protein (Table III) was determined for untreated membranes. This value was found to be higher than the value reported by Sato and his group (50.9 nmoles of sialic acid / mg of protein) (Sato *et al.*, 1987) but was in good agreement with the value reported by Choy *et al.* (1979). Neuraminidase treatment for 2 hours resulted in the release of about 77% of sialic acid whereas

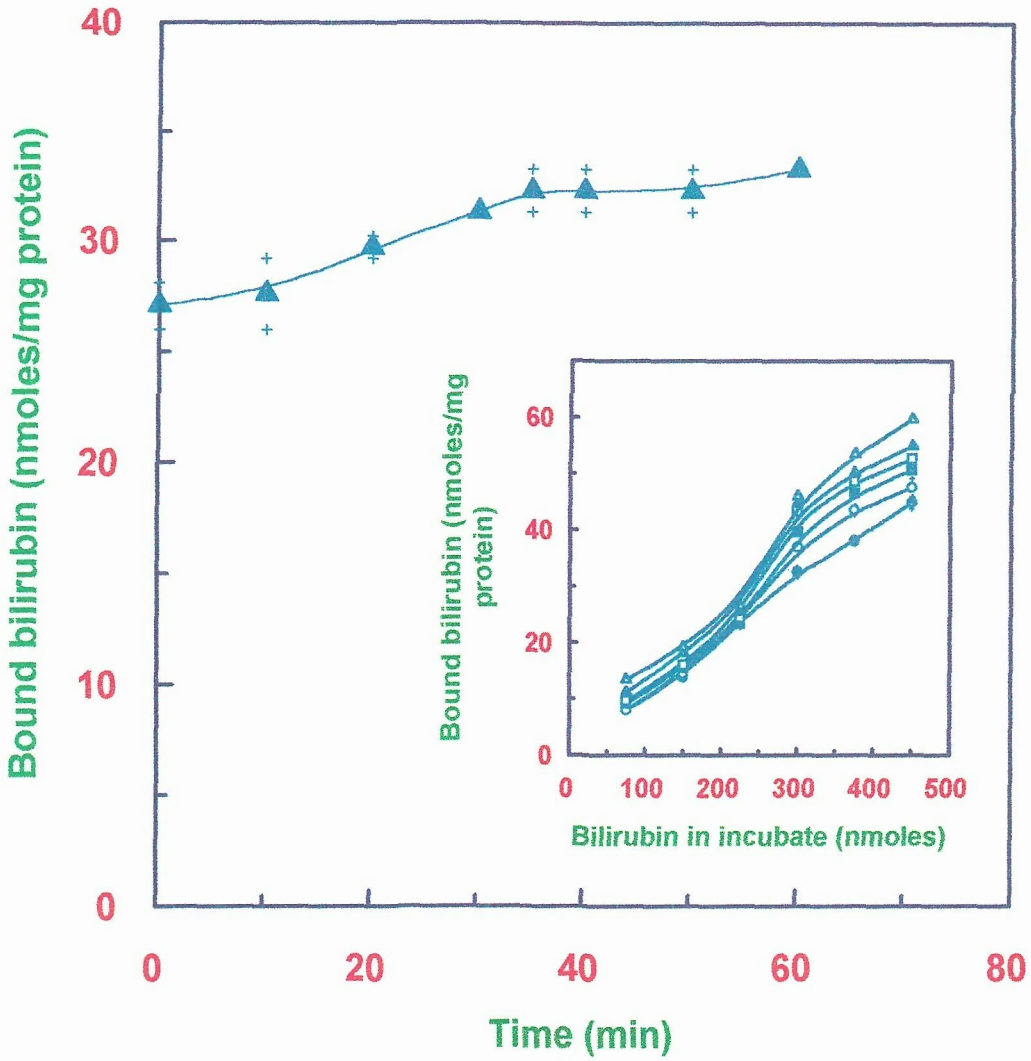


Figure 26. Effect of phospholipase D (Cabbage) treatment on binding of bilirubin. Amount of bilirubin bound to phospholipase treated membranes is shown by closed triangles (▲). Each point is mean of two independent experiments.

Inset shows the amount of bilirubin bound with increasing bilirubin concentration at different time periods of phospholipase D treatment, 0 min (•); 10 min (∘); 20 min (▪); 40 min (▫); 50 min (▴) and 60 min (▵).

Sato *et al.* (1987) reported the release of 88% of sialic acid upon neuraminidase treatment. The possible discrepancy between our results and those reported earlier can be ascribed to the lower values of sialic acid in untreated membranes obtained by Sato and his group (1987). Effect of neuraminidase treatment on human erythrocyte membranes, visualized by carbocyanin staining of polyacrylamide gels is seen in figure 28. The blue bands signifying the position of PAS bands 1, 2 and 3 disappear even within 5 min of neuraminidase treatment (Figure 28A) and these correspondingly appear in the supernatant fraction along with other polypeptides obtained after centrifugation of untreated and treated membrane suspensions (Figure 28B).

Bilirubin-bound membranes (preincubated with 450 nmoles of bilirubin at B/A of 2.0) produced the same results upon neuraminidase treatment as compared to those observed with control membranes. The only difference was observed in the initial phase (up to 20 min) of enzyme treatment where bilirubin-bound membranes showed some protection as judged by the lesser release of sialic acid from these membranes. It appears that the presence of bilirubin at the membrane surface offers some resistance to the enzymatic attack on some of the cleavage sites for the initial time period which also become accessible to the enzyme during prolonged enzyme treatment.

Both membrane preparations (control as well as neuraminidase-treated for various time periods) were used for bilirubin binding studies after incubation with 225 nmoles of bilirubin at B/A of 2.0 and the results are shown in figure 29. As can be seen from the figure, 1 hour of enzyme treatment resulted in 41%

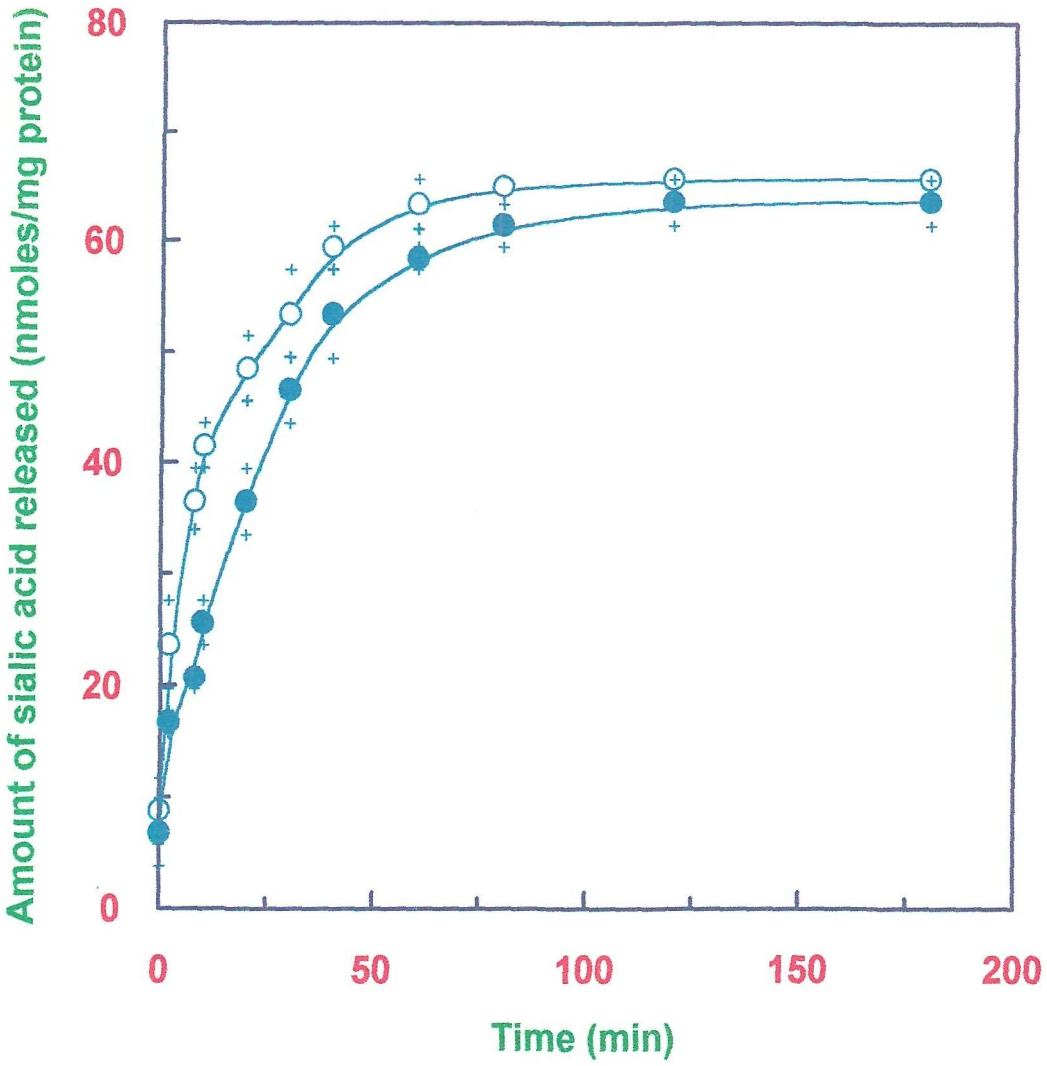


Figure 27. Time course of neuraminidase (*C. perfringens*) treatment of human erythrocyte membranes as monitored by sialic acid release. Open circles (○) show the amount of sialic acid released upon enzyme treatment from normal membranes whereas closed circles (●) represent sialic acid released from bilirubin-bound membranes. *Each point is the mean of two independent experiments.*

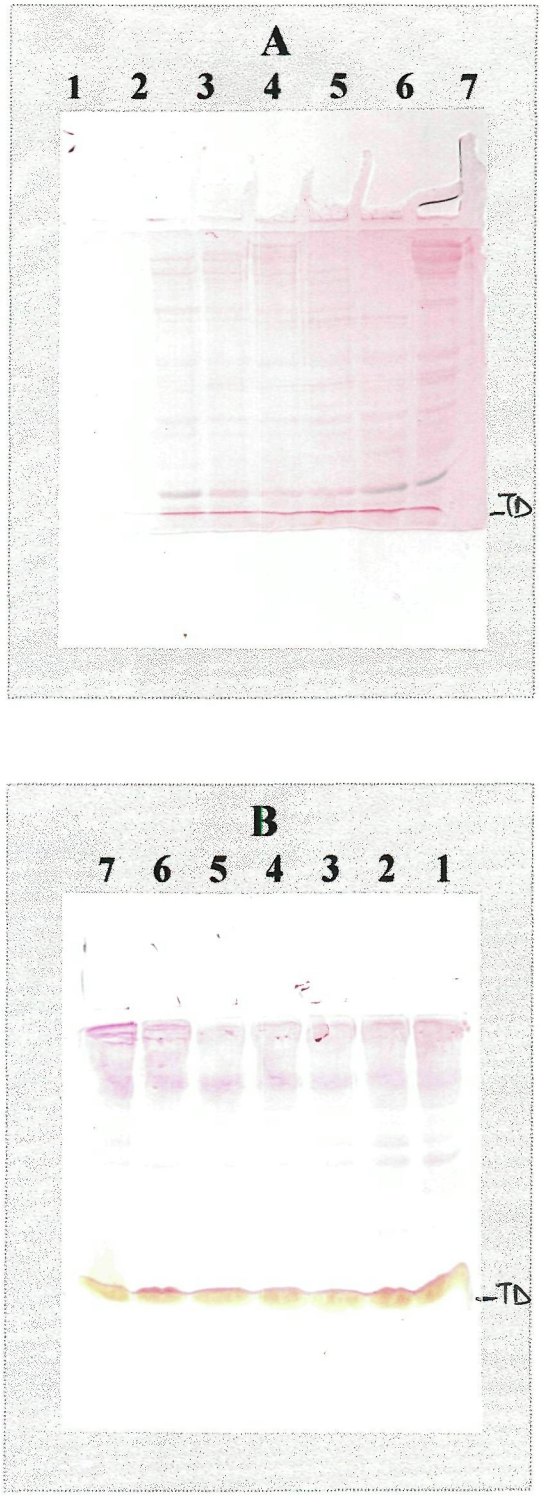


Figure 28. (A) Electrophorogram (SDS-PAGE) of the supernatant collected by centrifuging neuraminidase (*C. perfringens*)-treated membranes [5 min (1), 10 min (2), 20 min (3), 40 min (4), 60 min (5), 120 min (6) and 180 min (7)] at 16,000×g for 20 min at 4°C as visualized by carbocyanin staining.
(B) Electrophorogram (SDS-PAGE) of human erythrocyte membranes after 5 min (1), 10 min (2), 20 min (3), 40 min (4), 60 min (5) 120 min (6) and 180 min (7) of neuraminidase (*C. perfringens*) treatment as visualized by carbocyanin staining.

increase in the bound bilirubin compared to untreated membranes. This increase in bound bilirubin upon neuraminidase-treatment correlated to some extent with the release of sialic acid from membrane surface, which suggests some inhibitory role of sialic acid residues in the bilirubin-binding phenomenon to the membranes. Although these results are contradictory in terms of the increase in bilirubin binding upon neuraminidase-treatment with those of Sato and his group (1987) but agree very well in excluding the gangliosides as the candidates for bilirubin binding.

Trypsin treatment of erythrocyte membranes resulted in the release of glycopeptides, which was monitored by carbohydrate measurement. Results of tryptic digestion of erythrocyte membranes are shown in figure 30A, in which the amount of carbohydrate released is plotted against time of incubation of enzyme with the membrane. As is clear from the figure, there was significant increase in the amount of carbohydrate released on increasing the time of incubation up to 60 min beyond which it sloped off. As much as 74% of total membrane carbohydrate was released in 3 hours. When tryptic digestion of bilirubin-bound membranes (previously treated with 450 nmoles of bilirubin at B/A of 2.0) was performed under similar conditions, a slight decrease in the amount of carbohydrate released was noticed. This is understandable, as some of the tryptic cleavage sites of membrane proteins must have been protected by the bound bilirubin. Figure 31 shows the electrophoretic pattern of untreated and trypsin treated membranes for different time periods as visualized by coomassie and carbocyanin staining. Decrease in polypeptide bands (Figure 31A) and

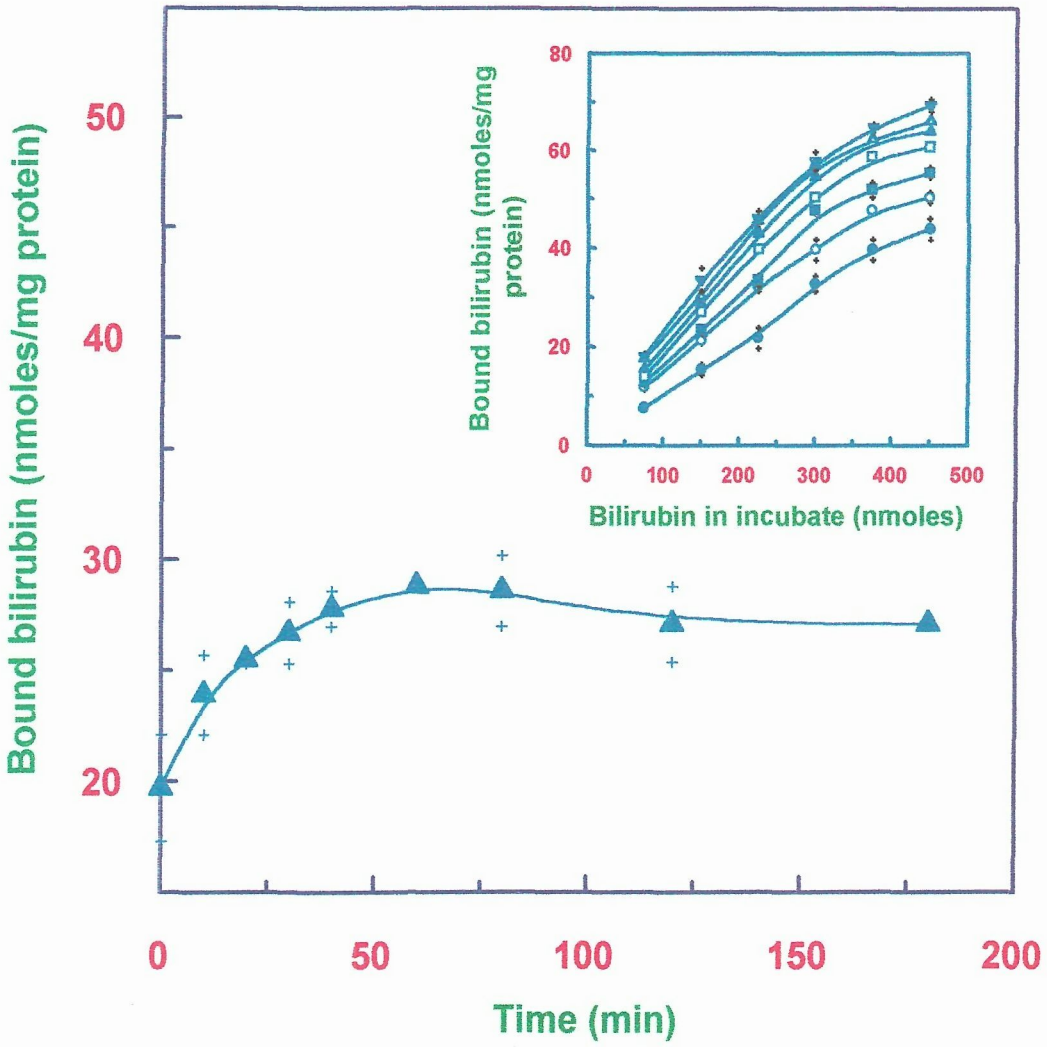


Figure 29. Effect of neuraminidase (*C. perfringens)* on the binding of bilirubin. Amount of bilirubin bound to neuraminidase-treated membranes is shown by closed triangles (▲). Each point is the mean of two independent experiments.
Inset shows the amount of bilirubin bound with increasing bilirubin concentration at different time periods of neuraminidase treatment, 0 min (•); 10 min (◦); 15 min (▪); 30 min (▫); 60 min (▴); 120min (▴) and 240 min (▾).

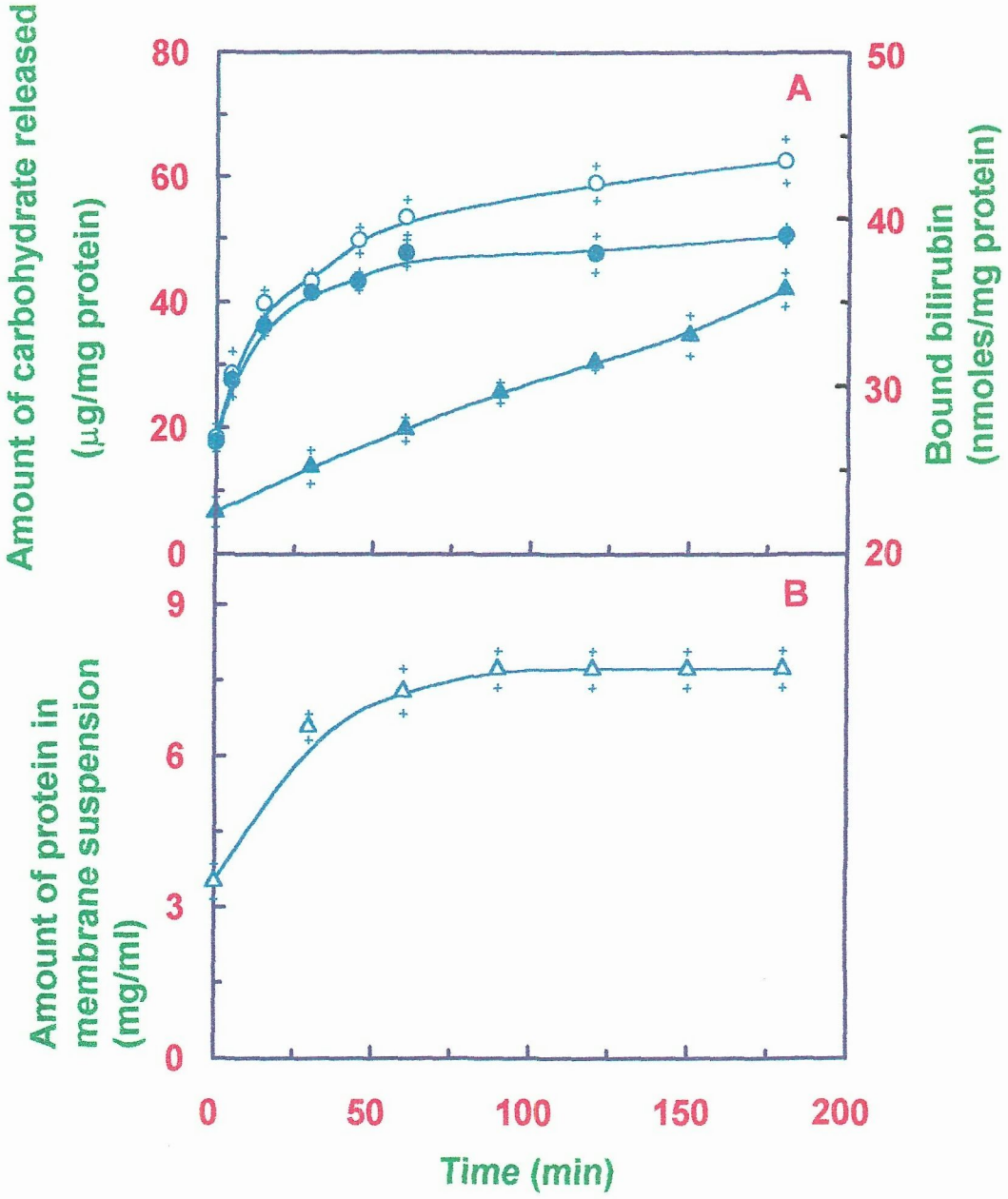
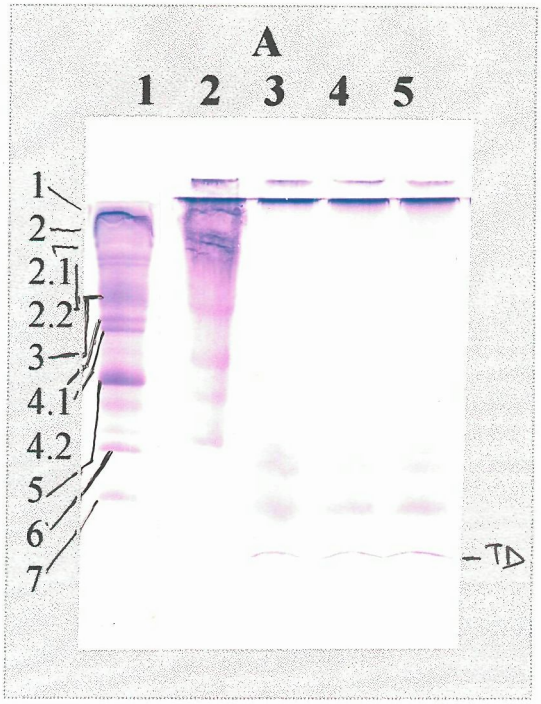
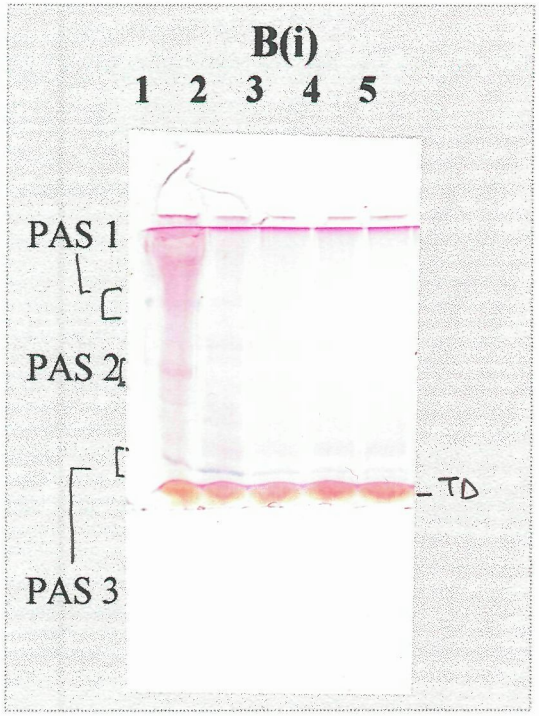


Figure 30. (A) Time course of trypsin treatment of human erythrocyte membranes as monitored by carbohydrate release and its effect on the binding of bilirubin. Open circles (○) show the amount of carbohydrate released upon enzyme treatment from normal membranes whereas closed circles (●) represent carbohydrate released from bilirubin-bound membranes. Amount of bilirubin bound to trypsin-treated membranes is shown by closed triangles (▲). Each point is the mean of two independent experiments.
(B) Binding of trypsin to human erythrocyte membranes as monitored by the measurement of protein content in the membrane suspension shown as open triangles (△). Each point is the average of two separate determinations.





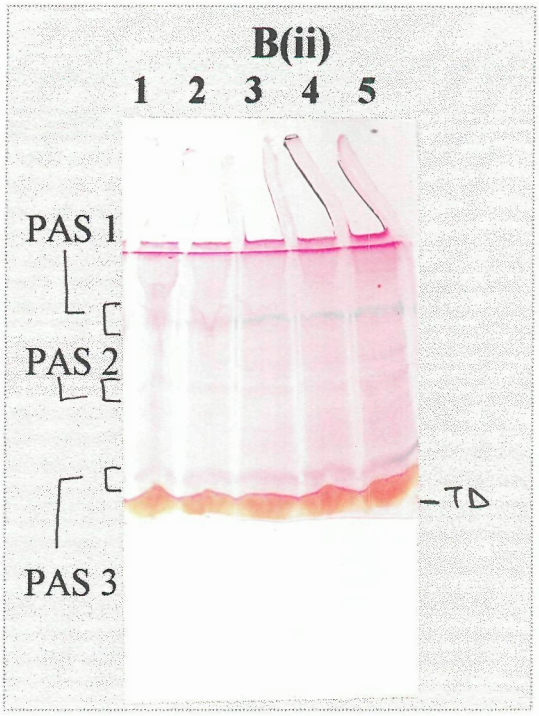


Figure 31. (A) SDS-PAGE analysis of human erythrocyte membrane before (1) and after 15 min (2), 30 min (3), 60 min (4) and 120 min (5) of trypsin treatment as visualized by (A) coomassie blue staining and **(B, i)** carbocyanin staining.
(B, ii) Electrophoretic pattern of the supernatant collected before (1) and after similar time periods of trypsin treatment as figure 31A by centrifuging at 16,000×g for 20 min at 4°C as visualized by carbocyanin staining.

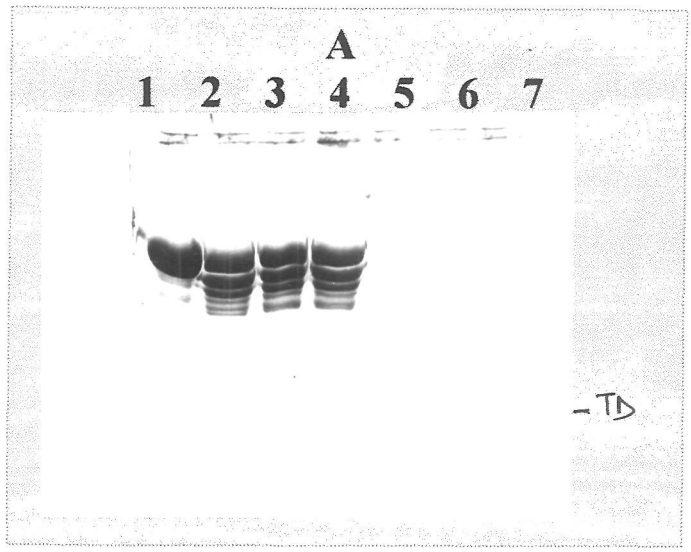
glycopeptides (Figure 31B, i) is visible by both coomassie and carbocyanin staining showing the effect of trypsin treatment of membranes for different time periods. A corresponding increase in the intensity of glycopeptide bands visualized as blue (Figure 31B, ii) is also evident from the figure in the supernatant fraction obtained by centrifugation at 16,000×g for 20 min at 4°C.

A significant increase in bilirubin binding to the membranes was noticed upon trypsin treatment when these treated membranes were incubated with 225 nmoles of bilirubin for 30 min. An analysis of bilirubin-binding data of treated membranes and untreated membranes showed about 57 % increase in the binding of bilirubin upon 3 hours trypsin treatment of the membranes (Figure 30A). These results were similar to those reported by Sato & Kashiwamata (1983) who suggested that the trypsin treatment of the membranes disclosed a great number of low-affinity bilirubin binding sites and that the bilirubin binding sites are not composed of proteins.

These conclusions seem to be questionable in view of the retention of activity of membrane-bound trypsin (Burkholder & Brecher, 1972) which may cleave the albumin present in the incubation medium, thereby, increase the free bilirubin concentration in the incubation mixture, being accessible to the membranes. To check this possibility, we estimated the protein content both in untreated and trypsin-treated membranes. As trypsin treatment of the membranes is supposed to degrade the membrane proteins, one should expect a decreased protein content in trypsin-treated membranes compared to untreated membranes. Contrary to this, an increase in the amount of membrane protein was observed upon trypsin

treatment (Figure 30B), which suggested the binding of trypsin to the cell membranes. If this is really the case then incubation of these trypsin-treated membranes (with bound enzyme) with bilirubin-albumin mixture at B/A of 2.0 for 30 min should degrade the albumin present in the medium.

When both untreated as well as trypsin-treated membranes were incubated with bilirubin-albumin mixture under the conditions of bilirubin binding experiment and the supernatants obtained after centrifugation was analysed by SDS-PAGE, significant degradation of albumin was observed as shown in figure 32A. Further, it should be noted that trypsin treatment of the membrane for more than 1 hour did not result in any increase in membrane-bound enzyme activity as similar albumin degradative pattern was observed with all the three trypsin-treated membranes (see figure 32A). This is also understandable from figure 30B in which no further increase in membrane protein was observed after 1 hour of enzyme treatment. In order to verify these results, 3 hours trypsin-treated membranes were incubated with increasing concentrations of albumin and the supernatants obtained after centrifugation were analysed by SDS-PAGE. The results are shown in figure 32B in which the intensity of low molecular weight degradative products was found to increase with the increase in albumin concentration in incubate. Taken together, all these results (Figures 30B, 32A and 32B) unequivocally suggest the degradation of albumin by the membrane-bound activity found in trypsin-treated membranes. Therefore, degradation of albumin in the incubation mixture will certainly increase the free bilirubin concentration in the incubation medium, which may be available to the trypsin-



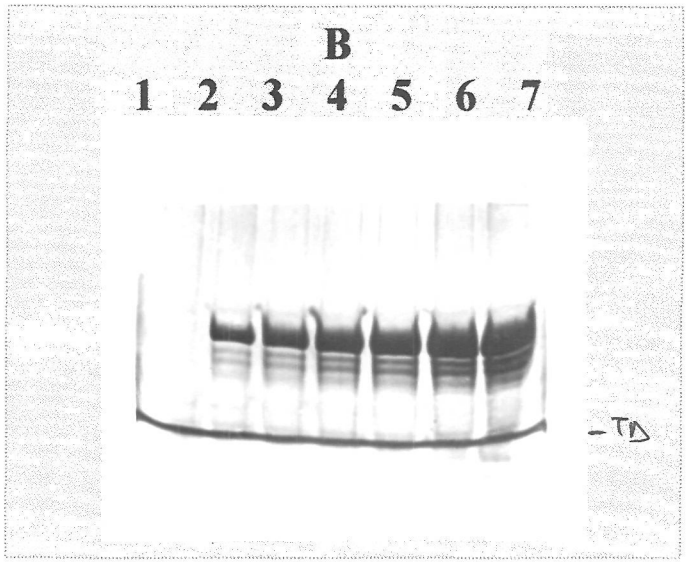


Figure 32. (A) SDS-PAGE analysis of the supernatant obtained by centrifugation of the incubation mixture containing untreated/trypsin-treated erythrocyte membranes and bilirubin-albumin mixture at B/A of 2.0. Lane 1 shows the result when untreated membranes were taken whereas lanes 2, 3 and 4 represent the results obtained with trypsin-treated (1, 2 and 3 h, respectively) membranes. Lanes 5, 6 and 7 show the result when trypsin-treated membranes were incubated with bilirubin in the absence of albumin.

(B) SDS-PAGE analysis of the supernatant obtained by centrifugation of the incubation mixture containing 3 h trypsin-treated erythrocyte membranes and increasing concentrations of albumin. Lanes 1-7 show the results obtained when the concentration of albumin in the incubation mixture was 0, 25, 50, 75, 100, 125, 150 μM, respectively.

treated membranes. In view of this, increased bilirubin binding to erythrocyte membranes upon trypsin treatment cannot be ascribed solely to the degradation of membrane proteins which have been suggested to act as a barrier in bilirubin binding to the membranes (Sato *et al.*, 1987). From our results it appears that removal of carbohydrate from membrane surface exposes more surface area of the membrane for bilirubin to interact.

The effect of Ca^{2+} ions on the binding of bilirubin to human erythrocyte membranes was studied after incubating the membranes with different concentrations of $CaCl_2$ in the range of 0-1.5 mM and then using these membranes after washing, for bilirubin-binding experiments. Incubation of Ca^{2+}-treated membranes with 150 nmoles of bilirubin at B/A of 2.0 for 30 min resulted in an increase in membrane-bound bilirubin as compared to the amount of bound-bilirubin observed with control membranes. This increase in membrane-bound bilirubin was dependent on $CaCl_2$ concentration as the binding increased on increasing the $CaCl_2$ concentration as shown in figure 33. As much as 85% increase in the amount of membrane-bound bilirubin was observed with 1.0 mM $CaCl_2$ treatment beyond which it sloped off. The increase in bilirubin binding upon $CaCl_2$-treatment can be ascribed to the shielding effect, redistribution of phospholipids as well as increase in surface hydrophobicity induced by calcium (Ito *et al.*, 1975). It seems likely that this effect may also be mediated by endogenous phospholipases as activation of phospholipase C in nonexcitable cells has been shown to cause the release of Ca^{2+} from intracellular stores (Kaznacheyeva *et al.*, 2001).

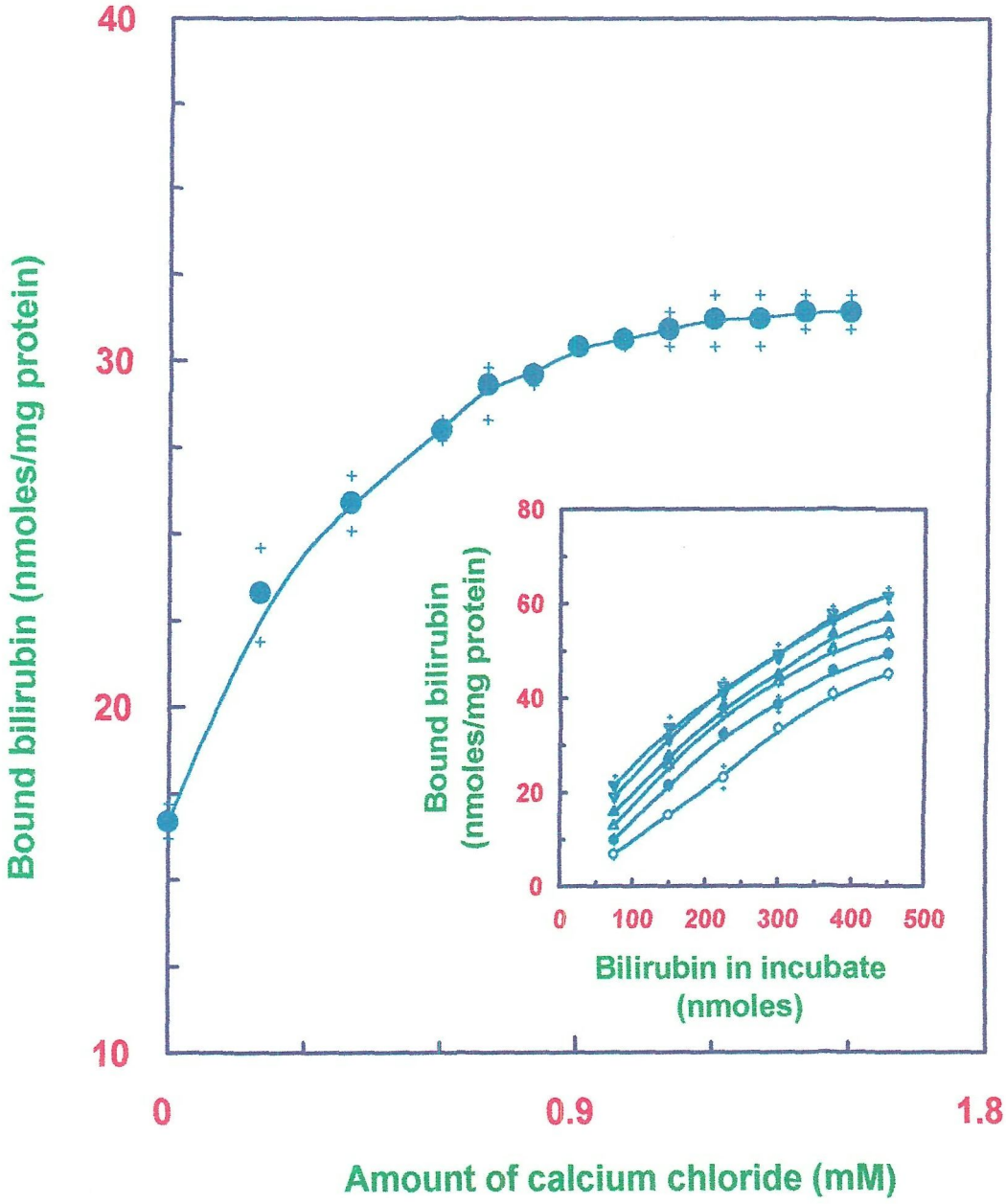
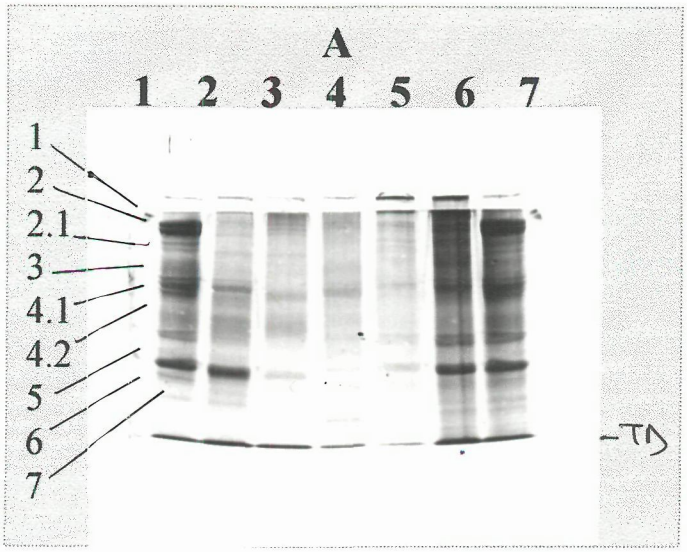


Figure 33. Effect of $CaCl_2$ treatment of human erythrocyte membranes on the binding of bilirubin (●). Each point is the average of two separate determinations.
Inset shows the amount of bilirubin bound to erythrocyte membranes with increasing concentration of bilirubin in incubate at different calcium chloride concentrations,0 mM (∘); 0.25 mM (•); 0.5 mM (▲); 0.75 mM (▲); 1.0 mM (▿) and 2.0 mM (▾)

In conclusion, these results suggest that polar head groups (phosphate in particular) of phospholipids along with carbohydrate and sialic acid produce hindrance in the binding of bilirubin to erythrocyte membranes. Further, binding of bilirubin to the membrane seems to be hydrophobic in nature.

Interaction of bilirubin with native and protein-depleted human erythrocyte membranes

Incubation of human erythrocyte membranes with alkaline ethylenediamine tetraacetic acid (EDTA) (0.1 mM, pH 8.0) resulted in the selective release of peripheral membrane proteins, namely, bands 1 and 2 as well as band 5 as shown in lane 2 of figure 34A and B. These results were in line with the previous reports (Kahlenberg, 1972; Fairbanks *et al.*, 1971). Measurement of protein in the pellets obtained from native and EDTA-treated membranes suggested a release of 32 % of proteins upon EDTA treatment (see table V). Conformational analysis of erythrocyte membranes, both before and after EDTA treatment as studied by CD spectroscopy suggested significant retention of the CD spectral features in EDTA-treated membranes (see figure 35A and B and table VI). However, some additional CD bands at 205 and 208 nm appeared in modified membranes. Further, a slight decrease in the MRE values of the CD band minima (Table VI) were noticed in these membranes. Presence of minima around 223 and 208 nm were indicative of α-helical structure of membrane proteins. Binding of BR to these membranes (both native and modified) was studied both by quantification of membrane-bound BR and CD spectroscopy. A comparison of the values of



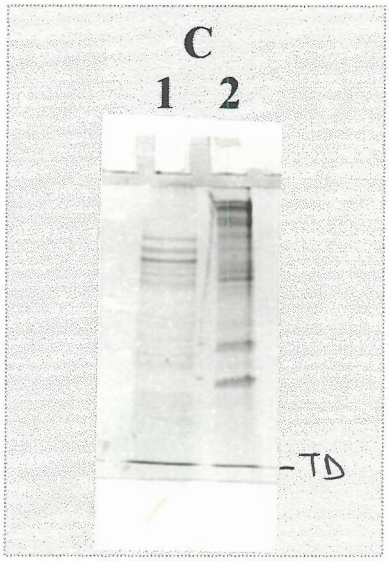


Figure 34. (A) Protein profiles of human erythrocyte membranes before (lane 1) and after treatment with 0.1 mM EDTA, pH 8.0 (lane 2); 1.0 M NaI (lane 3); 20 mg/ml DMMA (lane 4); 20 mM LIS (lane 5); 100 µM phenanthroline + 20 µM cupric sulfate (lane 6) and phenanthroline + cupric sulfate containing 100 µM 2-mercaptoethanol (lane 7).

(B) Protein profiles of released polypeptides as observed in the supernatant of native and treated human erythrocyte membranes. Various lanes represent the same order as shown in (A).

(C) Protein profiles of 0.1 mM NaOH-treated erythrocyte membranes (lane 1) and released polypeptides (lane 2).

About 25-35 µg of proteins were analysed electrophoretically on 10 % SDS - Polyacrylamide gels. TD is the position of tracking dye

membrane-bound BR obtained with native and EDTA-treated membranes suggested a small decrease (3%) in the membrane-bound BR upon EDTA treatment. Further, CD spectral analysis of the complexes of BR with these membranes showed more or less similar structural features with a red shift in one of the band minima and small decrease in ellipticity values (figure 35A and B, table VI). It can be interpreted from these results that the removal of peripheral proteins from the membranes did not affect the binding of BR significantly though, a slight alteration in membrane composition was observed as reflected from the significant red shift of the band minima at 214 nm. It should be mentioned that the removal of spectrin from the membrane may lead to destabilization of the membrane structure (Steck and Kant, 1973) which may account for the small decrease in BR binding and slight alteration in the CD signals.

Electrophoretic analysis of the membrane pellet obtained as a result of alkali treatment of human erythrocyte membranes (see figure 34C, lanes 1 and 2) show the release of about 64 % (Table V) of membrane proteins. It appears that most of the extrinsic proteins are eluted with sodium hydroxide (NaOH) treatment since a number of protein bands were visible in the electrophoretic profile of the supernatant (see lane 2 of figure 34C). The CD spectrum of NaOH-treated membranes (Figure 35C) showed a significant decrease in ellipticity at band minima (223 nm) whereas a blue shift of 4 nm was observed in the band minima at 212 nm and appearance of a new band at 203 nm (Table VI). Decrease in ellipticities at 223 nm was more pronounced in NaOH-treated membranes

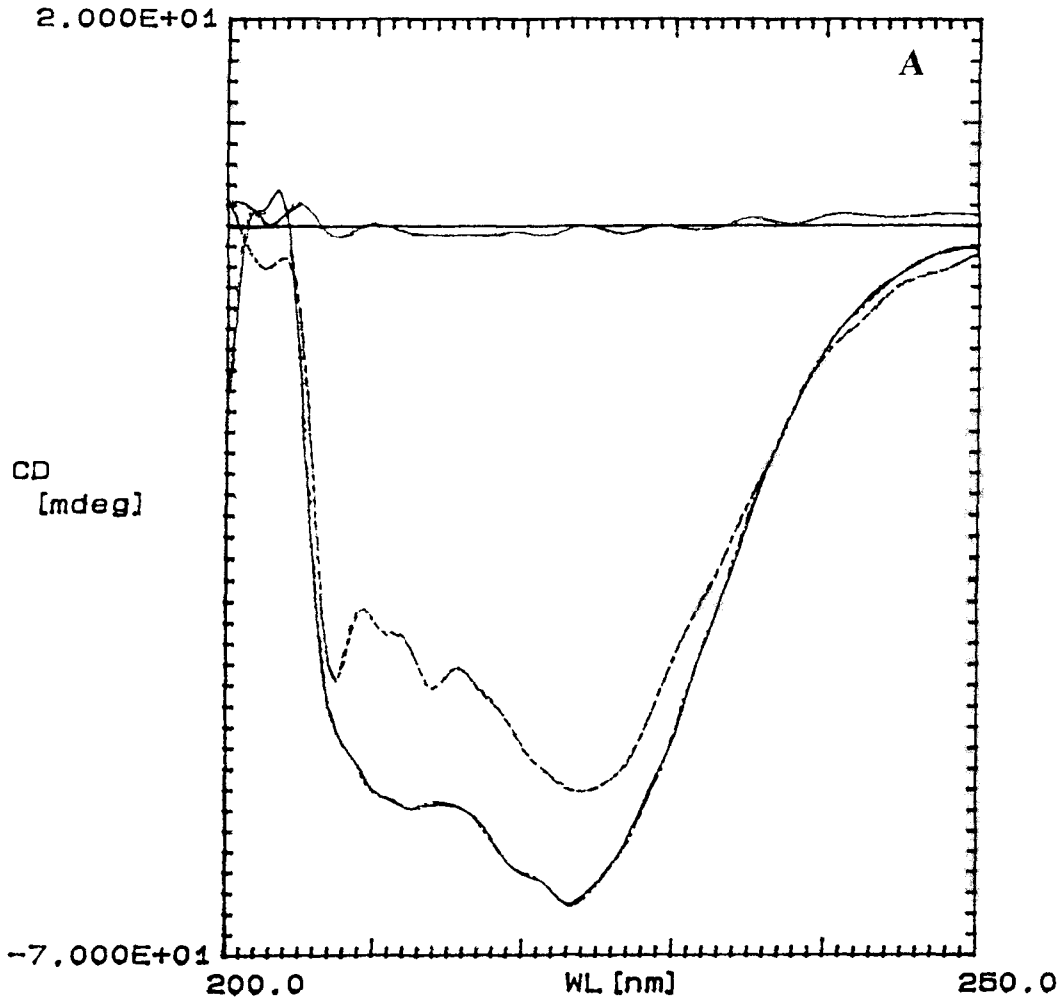
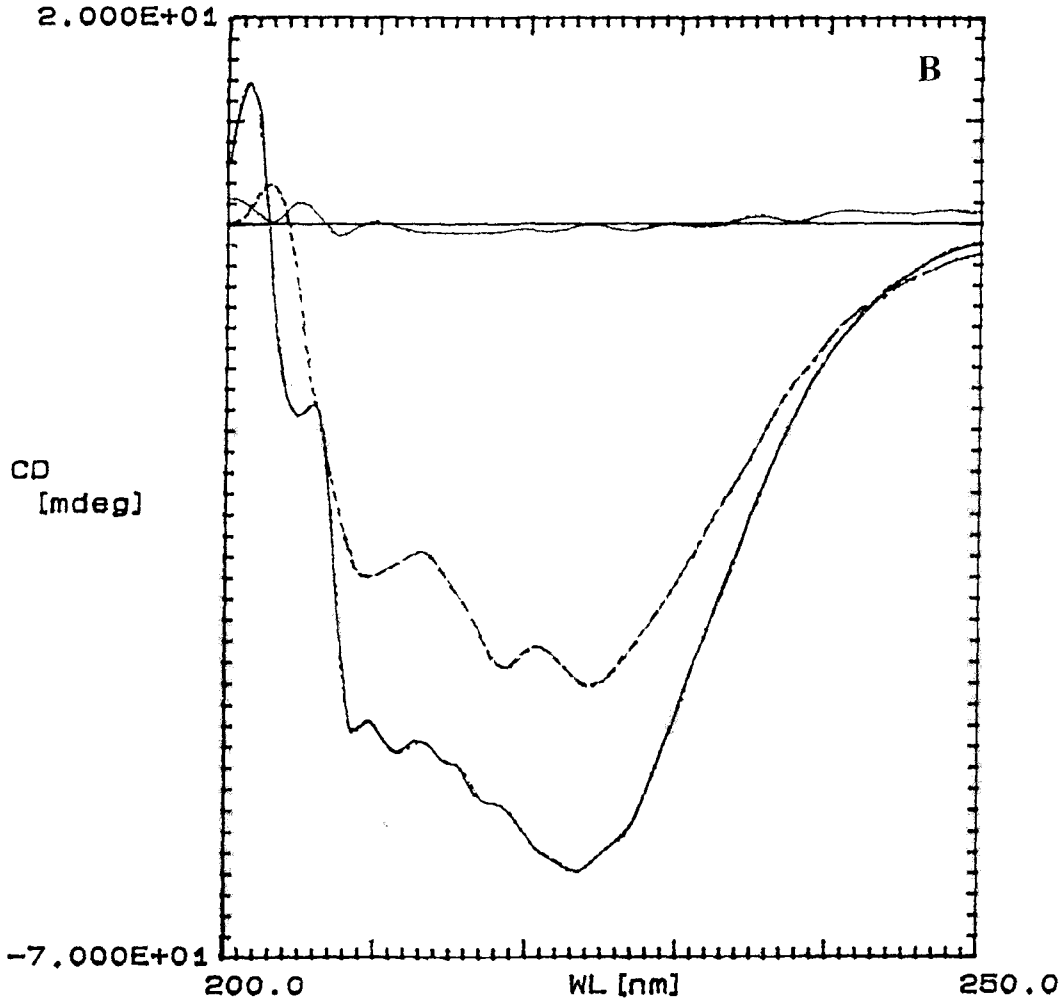


Figure 35. Far-UV CD spectra of human erythrocyte membranes before (solid line) and after the addition of 150 μM bilirubin (dotted line) in 5 mM sodium phosphate buffer, pH 7.4 at a protein concentration of 0.513 mg/4ml. Various membrane preparations were:
Native (A)
Treatment conditions were the same as described in the legend to figure 34.



Far-UV CD spectra of human erythrocyte membranes before (solid line) and after the addition of 150 μM bilirubin (dotted line) in 5 mM sodium phosphate buffer, pH 7.4 at a protein concentration of 0.513 mg/4ml. Various membrane preparations were:

EDTA-treated (B)

Treatment conditions were the same as described in the legend to figure 34.

Table V

Effect of treatment of erythrocyte membranes with selective reagents on their protein content and bilirubin binding

Membrane sample	Protein concentration in pellet		Membrane-bound bilirubin
	(mg/ml)	*%*	*(nmoles)*
NATIVE	3.08±0.19	100	114.0±0.82
EDTA-treated	2.10±0.27	68	110.6±0.83
NaOH-treated	1.12±0.14	36	99.6±0.50
LIS-treated	0.94±0.17	31	107.9±1.34
[a]CuP-treated	2.37±0.14	77	105.3±0.66
[b]CuP-mercaptoethanol treated	2.19±0.19	71	110.6±0.93
DMMA treated	1.29±0.17	42	119.3±1.26
NaI-treated	1.47±0.17	48	115.9±0.80

Each value is the mean of three individual experimental values.
[a]CuP refers to o-phenanthroline-cupric sulfate cross-linked membranes.
[b]CuP-mercaptoethanol refers to o-phenanthroline-cupric sulfate cross-linked membranes reduced with 2-mercaptoethanol.

Table VI

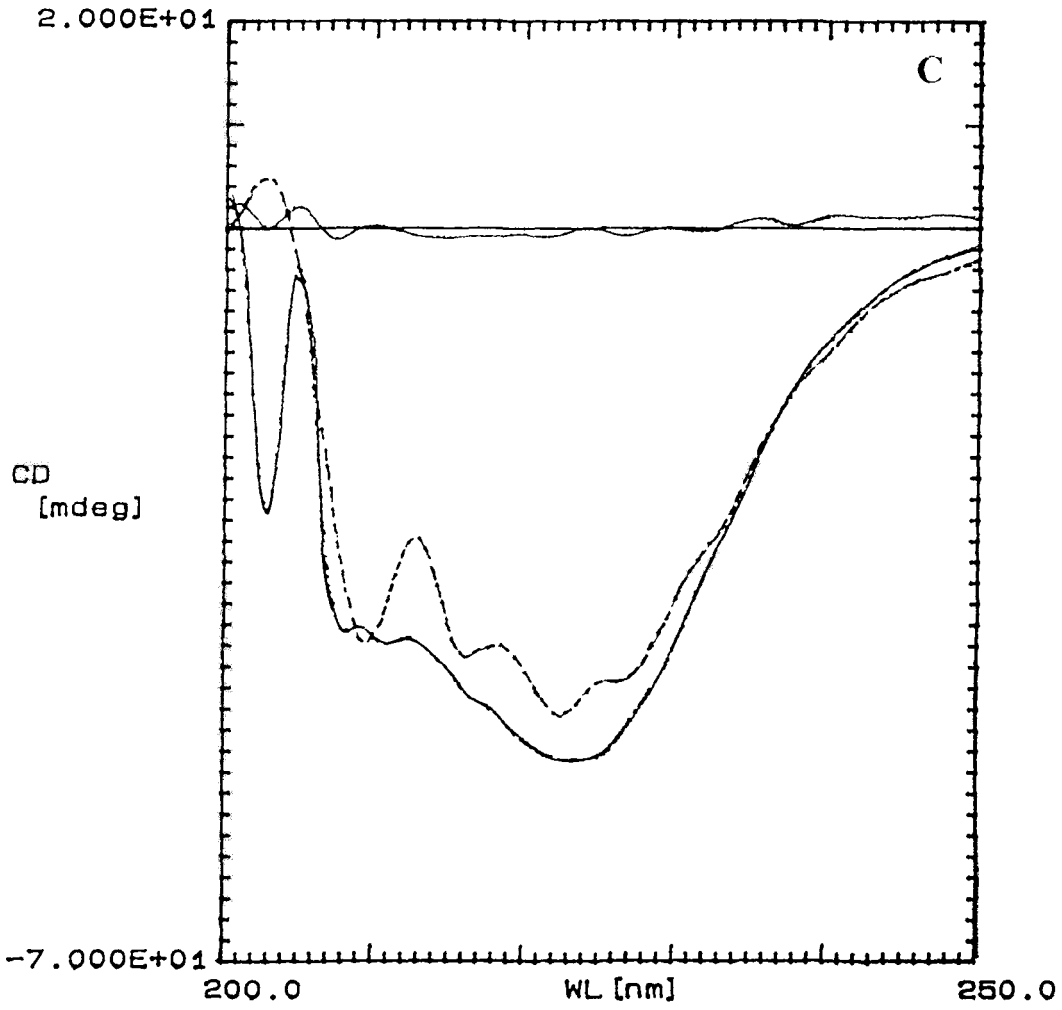
Circular dichroism data of native and treated human erythrocyte membranes before and after bilirubin binding

Human erythrocyte membrane	Observed CD band minima/maxima. Position (nm) and corresponding molar ellipticities in parenthesis *(deg cm^2 $dmol^{-1}$)*			
	Bilirubin (-)		*Bilirubin (+)*	
NATIVE	223	(-14710)	224	(-12030)
	212	(-12480)	214	(-9800)
			208	(-9800)
EDTA-treated	223	(-13820)	224	(-9800)
	212	(-11140)	218	(-9360)
	208	(-10700)	209	(-7580)
	205	(-4010)		
NaOH-treated	223	(-11140)	227	(-9800)
	208	(-8470)	223	(-10250)
	203	(-6240)	216	(-9360)
			209	(-8910)
LIS-treated	223	(-5120)	227	(-4460)
	218	(-5120)	221	(-4010)
	209	(-5350)	216	(-3570)
	205	(-2670)	214	(-3340)
			209	(-4900)
CuP-treated	224	(-13370)	224	(-11140)
	208	(-13370)	208	(-11140)
CuP-	223	(-13810)	226	(-10250)
mercaptoethanol	209	(-11590)	222	(-10700)
			218	(-9800)
			212	(-9360)
			207	(-10250)
DMMA-treated	239	(-2670)	239	(+4900)
	233	(-4900)	233	(+5570)
	227	(-2450)	228	(-1120)
			225	(-3340)
NaI-treated	247	(-1670)	246	(-780)

Presence and absence of bilirubin is represented as (+) and (-), respectively.

compared to EDTA-treated membranes, suggesting gross conformational changes in NaOH-treated membranes. BR binding studies to these membranes showed a significant decrease (13 %) in BR binding (Table VI). This decrease in BR binding can also be seen from the CD spectra of BR-membrane complexes in which the ellipticity at 209 nm was found to be similar to that obtained with treated membranes in the absence of BR. Presence of additional features at 227 and 216 nm and abolishment of minima at 203 nm in the presence of BR indicated conformational alterations in the membranes (Figure 35C, table VI). A significant decrease in BR binding observed with NaOH-treated membranes seems to be understandable in view of the depletion of extrinsic polypeptides namely bands 1, 2, 5 and 6 as well as predominant inside-out orientation of these vesicles upon dilute alkali treatment (Jones & Nickson, 1981). This is because inside-out vesicles have been shown to bind lesser amount of BR as compared to right side-out vesicles (Rashid *et al.*, 2000).

Treatment of membranes with 3,5-diiodosalicylic acid, lithium salt (LIS) resulted in the release of a majority of polypeptides (lane 5 of figure 34A and B), which together constituted 69 % of protein (Table VI). These results were in accordance with earlier reports suggesting the release of bands 1, 2, 2.1, 4.2, 5 and 6 from the membranes upon LIS treatment (Kahlenberg, 1972). LIS breaks the membrane into small vesicles and in addition to disrupting membranes it releases glycoproteins from the membranes (Kahlenberg, 1972; Marchesi & Andrews, 1971). Conformational analysis of LIS-treated membranes also revealed a marked decrease in ellipticity values at the band minima (223 nm) and



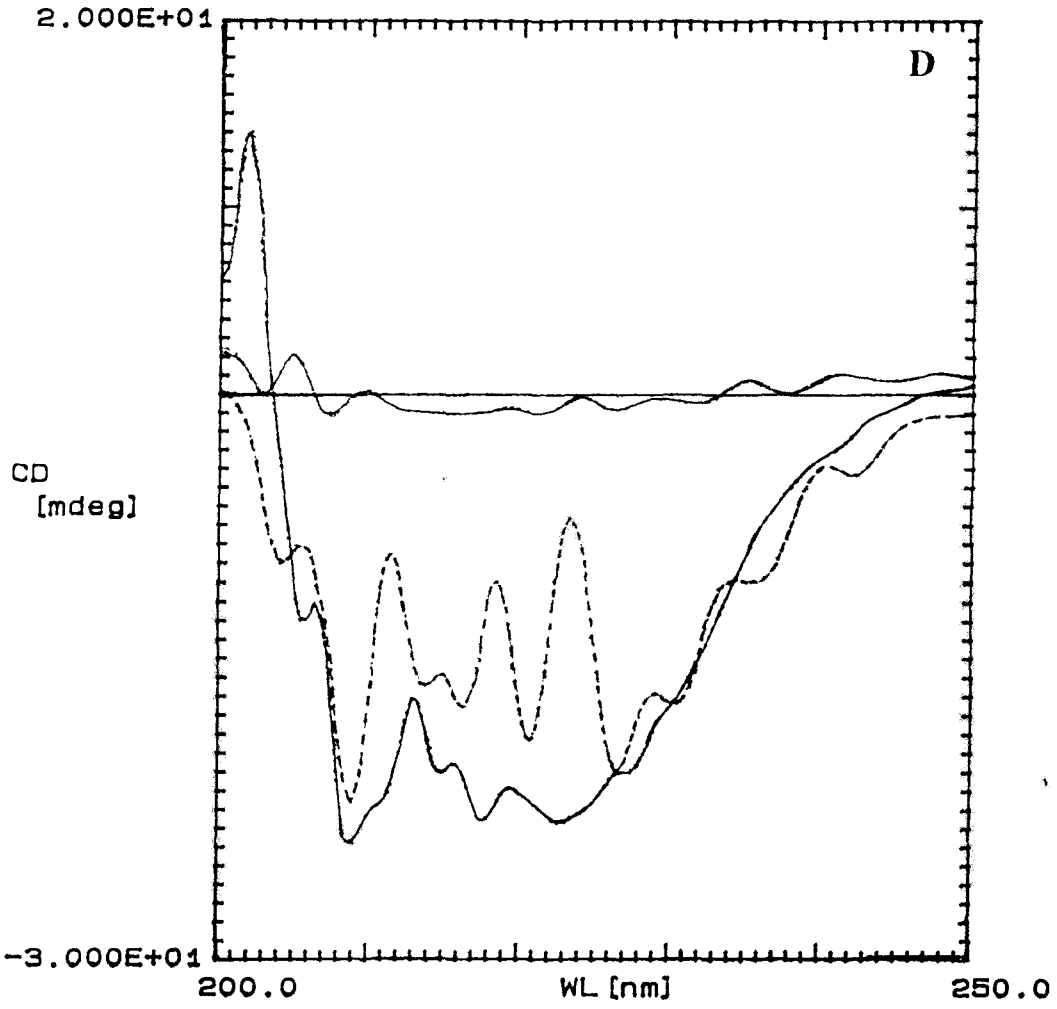
Far-UV CD spectra of human erythrocyte membranes before (solid line) and after the addition of 150 μM bilirubin (dotted line) in 5 mM sodium phosphate buffer, pH 7.4 at a protein concentration of 0.513 mg/4ml. Various membrane preparations were:

NaOH-treated (C)

Treatment conditions were the same as described in the legend to figure 34.

appearance of more CD signals in comparison to untreated membranes (Figure 35D, table VI). These changes in the CD spectra can be attributed to the marked alteration in membrane conformation upon release of various polypeptides. Contrary to this, when LIS-treated membranes were studied for BR binding, only 6 % decrease in BR binding was noticed (Table V). Similarly, CD spectra of BR-membrane complex, though showed the appearance of additional peaks but the ellipticity values were significantly decreased (Table VI). Greater retention in BR binding by LIS-treated membranes compared to NaOH-treated membranes suggest that some other factors (other than protein loss) were responsible for the decrease in BR binding to the membranes.

In order to study the role of band 3 in the BR binding phenomenon, erythrocyte membranes were treated with o-phenanthroline and copper sulfate, (o-phenanthroline-cupric sulfate, CuP). This treatment resulted in the cross-linking of band 3 proteins as evident from lane 6 of figure 34A in which the band 3 protein was shifted to a higher molecular weight form. Also bands 1 and 2 were found to be cross-linked to a certain extent (see lane 6 of figure 34A). These results were in accordance with the earlier observations suggesting the cross-linking of largely band 3 and bands 1 and 2 by an o-phenanthroline-cupric sulfate catalysed oxidation (Kahlenberg, 1972). About 23 % of the membrane proteins were lost in cross-linking experiments (Table V). Kahlenberg (1972) also showed a 25 % loss of ghost protein in their cross-linking experiments. CD spectra of cross-linked membranes showed strong α-helical characteristics as evident by the presence of two band minima at 224 and 208 nm with a slight decrease in



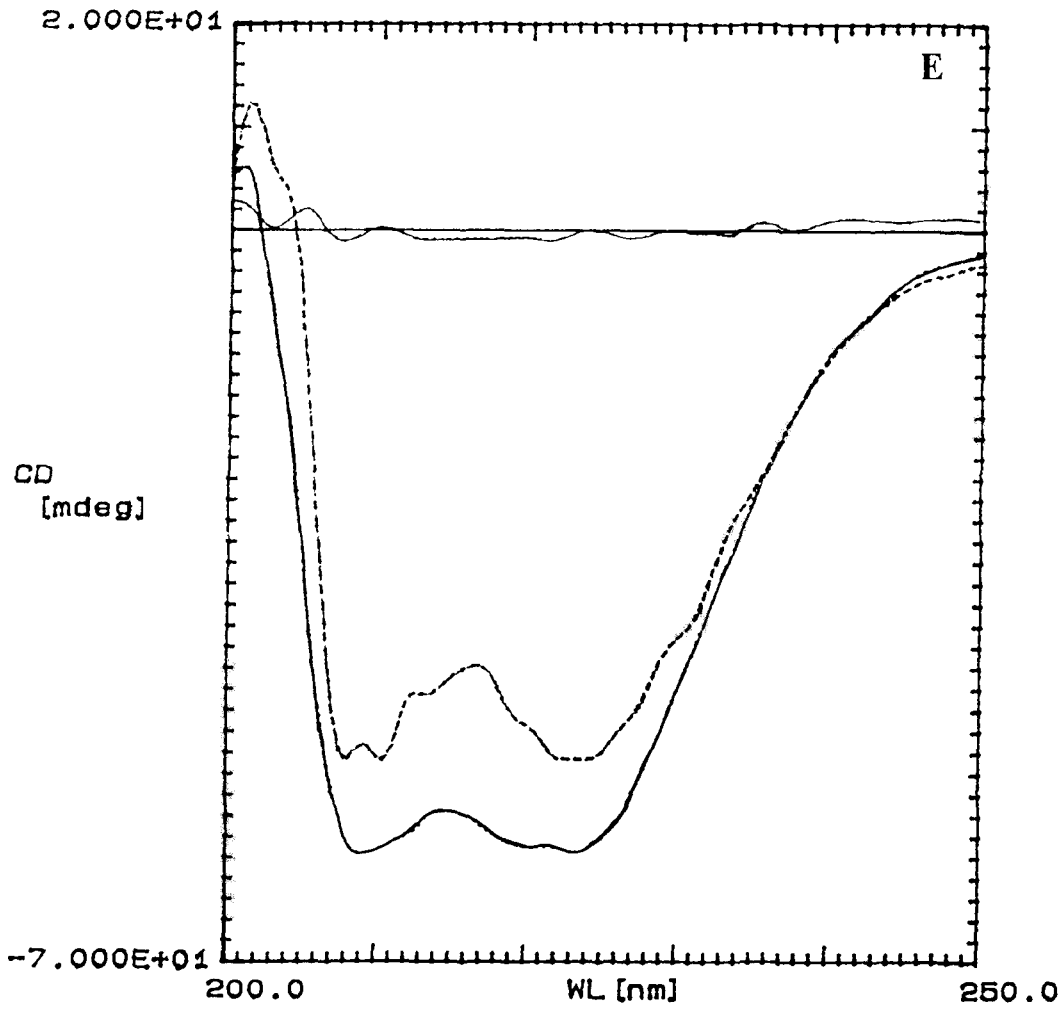
Far-UV CD spectra of human erythrocyte membranes before (solid line) and after the addition of 150 μM bilirubin (dotted line) in 5 mM sodium phosphate buffer, pH 7.4 at a protein concentration of 0.513 mg/4ml. Various membrane preparations were:

LIS-treated (D)

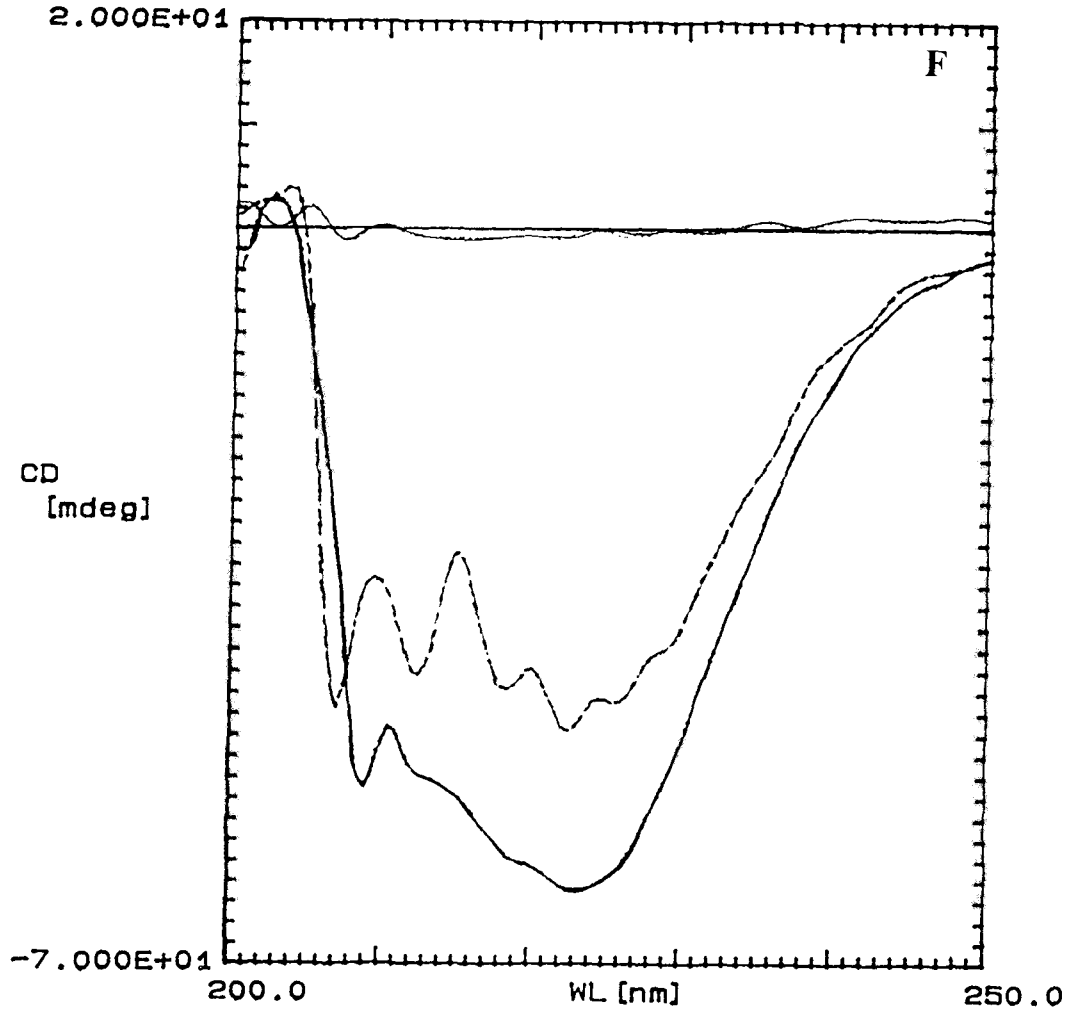
Treatment conditions were the same as described in the legend to figure 34.

ellipticity values (Table VI) as compared to native membranes. BR binding results obtained with these membranes indicated a smaller decrease (8 %) in BR binding as compared to native membranes. CD spectra of cross-linked membrane in the presence of BR also showed CD signals and ellipticity values comparable to native membrane (Figure 35E, table VI).

The decrease in BR binding upon cross-linking of membrane proteins can be ascribed to either the loss of some membrane proteins or the formation of larger aggregates. To rule out the first possibility, reversal of the cross-linking reaction was carried out by reduction with 2-mercaptoethanol. That phenanthroline-copper induced cross-linking is prevented in the presence of 2-mercaptoethanol is evident from the protein profile of native membrane with reference to band 3 protein (lane 7 of figure 34A). However, 29 % loss (Table V) in protein content was observed upon this treatment, which was 6 % higher than the loss observed with cross-linking treatment in the absence of 2-mercaptoethanol. CD spectra of these membranes also showed more or less similar features with a slight decrease in ellipticity at 209 nm to those observed with cross-linking experiments (Figure 35F, table VI). Presence of 2-mercaptoethanol resulted in the recovery of BR binding as these membranes showed 97 % binding of BR compared to 92 % observed with cross-linked membranes (Table V). This can also be seen from the CD spectra of the complexes of these membranes with BR (Figure 35F, table VI). Thus, it appears that the decrease in BR binding, noticed in cross-linked membranes, was due to the formation of larger aggregates rather than the loss of proteins.

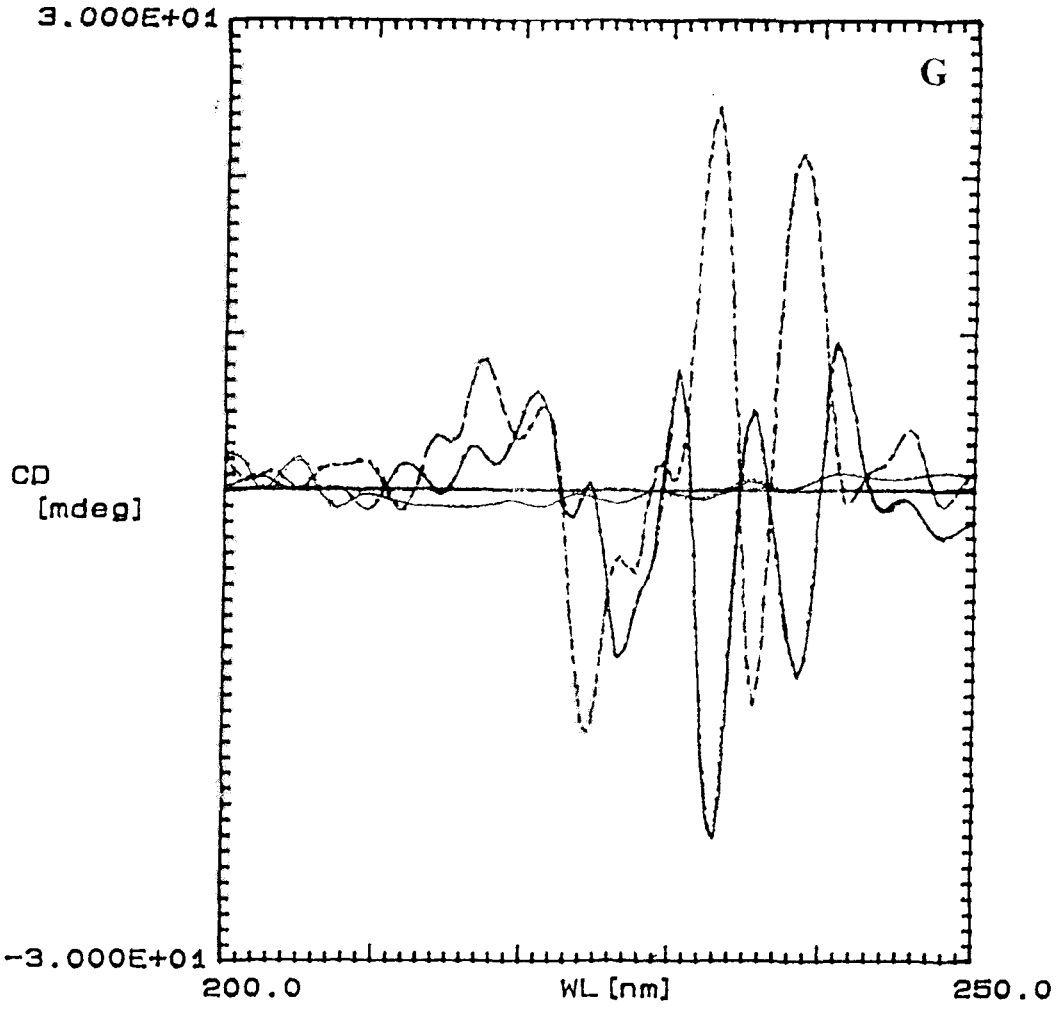


Far-UV CD spectra of human erythrocyte membranes before (solid line) and after the addition of 150 μM bilirubin (dotted line) in 5 mM sodium phosphate buffer, pH 7.4 at a protein concentration of 0.513 mg/4ml. Various membrane preparations were:
phenanthroline + cupric sulfate-treated (E)
Treatment conditions were the same as described in the legend to figure 34.



Far-UV CD spectra of human erythrocyte membranes before (solid line) and after the addition of 150 μM bilirubin (dotted line) in 5 mM sodium phosphate buffer, pH 7.4 at a protein concentration of 0.513 mg/4ml. Various membrane preparations were:
phenanthroline + cupric sulfate + 2-mercaptoethanol-treated (F)
Treatment conditions were the same as described in the legend to figure 34.

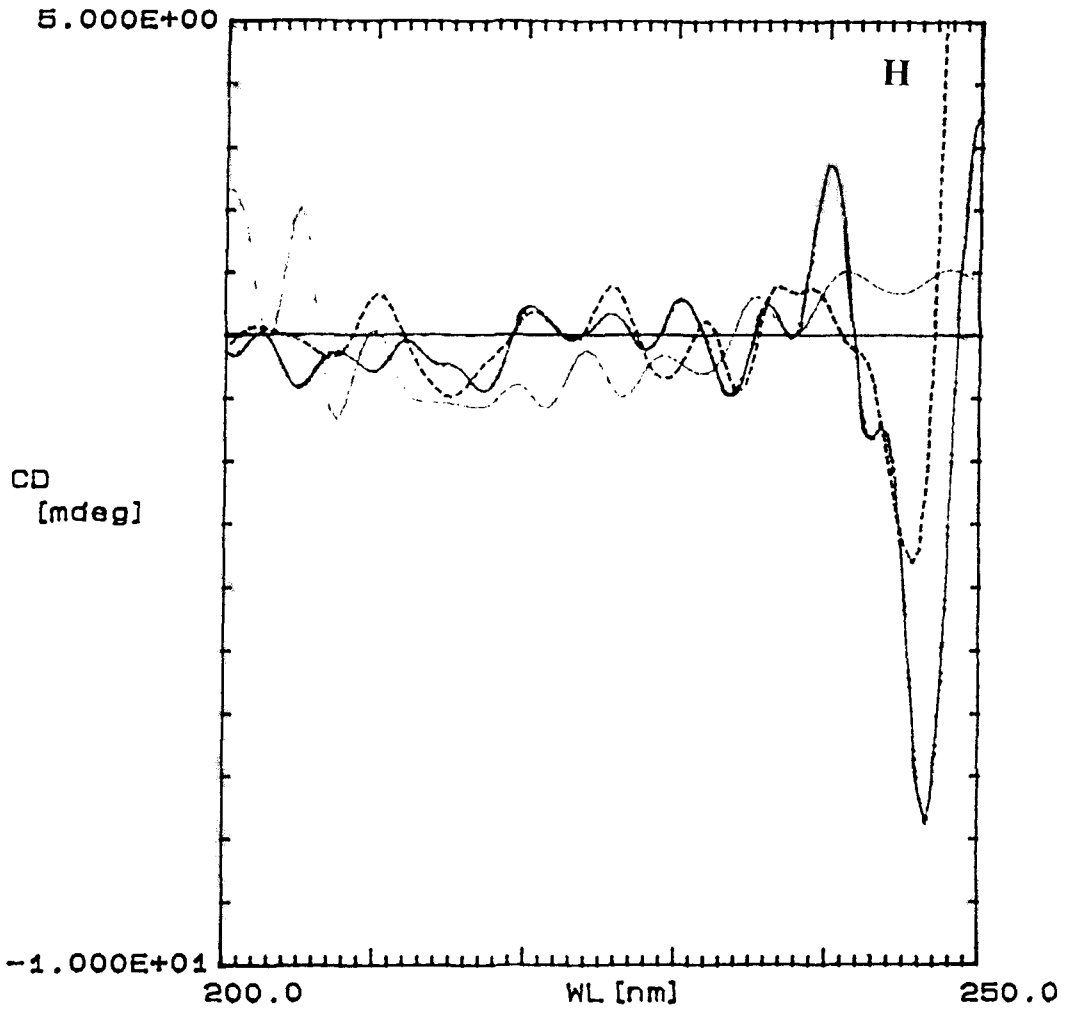
Selective removal of the membrane proteins was also carried out by dimethylmaleic anhydride (DMMA) and sodium iodide (NaI) treatments. As can be seen from table V, about 58 % and 52 % proteins were released by DMMA and NaI treatments, respectively. This can also be seen from protein profiles of these membranes shown in lanes 3 and 4 of figure 34A and B, respectively. Similar loss of membrane proteins by these treatments has also been reported earlier (Kahlenberg, 1972). Majority of the proteins eluted by these treatments were peripheral proteins as shown in lane 3 and 4 of figure 34B. CD spectral analysis of these membranes showed unusual spectral features as bands were found to be red shifted towards the higher wavelength side (figure 35G and H as well as table VI). When these membranes were tested for BR binding slightly higher binding was noticed as compared to native membranes (Table V). CD spectra of the complexes were of complicated type. From these results it appears that the loss of proteins from these membranes by DMMA and NaI treatment changed the overall conformation of band 3 as the CD spectra of these membranes were totally different from those of LIS-treated membranes in which about 69 % of the total proteins were released helical spectral features were retained as shown by native membranes. This unusual perturbation in the membrane may account for the complete retention rather little higher BR binding properties as compared to native membranes. The disappearance of double minima reflects the loss of α-helical structure and the transition towards random coil. It can be assessed that the disruption of self-associated band 3 by DMMA is accompanied with a disruption of helical structure, thereby giving the impression



Far-UV CD spectra of human erythrocyte membranes before (solid line) and after the addition of 150 μM bilirubin (dotted line) in 5 mM sodium phosphate buffer, pH 7.4 at a protein concentration of 0.513 mg/4ml. Various membrane preparations were:

DMMA-treated (G)

Treatment conditions were the same as described in the legend to figure 34.



Far-UV CD spectra of human erythrocyte membranes before (solid line) and after the addition of 150 μM bilirubin (dotted line) in 5 mM sodium phosphate buffer, pH 7.4 at a protein concentration of 0.513 mg/4ml. Various membrane preparations were:

NaI-treated (H)

Treatment conditions were the same as described in the legend to figure 34.

that the CD spectrum of membranes was largely due to the folded structure maintaining a band 3-dimer (Makino & Nakashima, 1982).

Taken together, all these results suggest that removal of membrane proteins does not affect the BR binding properties of erythrocyte membranes to an appreciable extent. These results strengthen previous findings (Sato & Kashiwamata, 1983; Nagaoka & Cowger, 1978; Cestaro *et al.*, 1983; Vazquez *et al.*, 1988) suggesting that BR binding sites in the membranes are composed of lipids rather than proteins.

Interaction of bilirubin with sealed and human serum albumin-entrapped sealed membranes

Free membrane and bilirubin (BR) exhibit no circular dichroism in the visible wavelength range of 350-550 nm (Gray *et al.*, 1961) as seen from figure 36a and b, respectively. This is similar in case of free albumin and free drug (ceftriaxone and sodium salicylate), exhibiting no CD at wavelength above 300 nm however, bilirubin-bound sealed membrane (BR-sealed membrane) complex in the visible-wavelength range of 350-550 nm shows bisignate CD spectra (Figure 37A) characterized by a positive CD cotton effect (CDCE) at higher wavelength and a negative CDCE at shorter wavelength. These observations are similar to those reported earlier (Lightner *et al.*, 1986; Lightner *et al.*, 1988) suggesting that the pigment binds to membrane in a folded conformation with positive chirality. BR-protein complex gives biphasic spectra in the range 300-500 nm. The induced bisignate CDCE of BR-membrane complex (Figure 37A) for the long

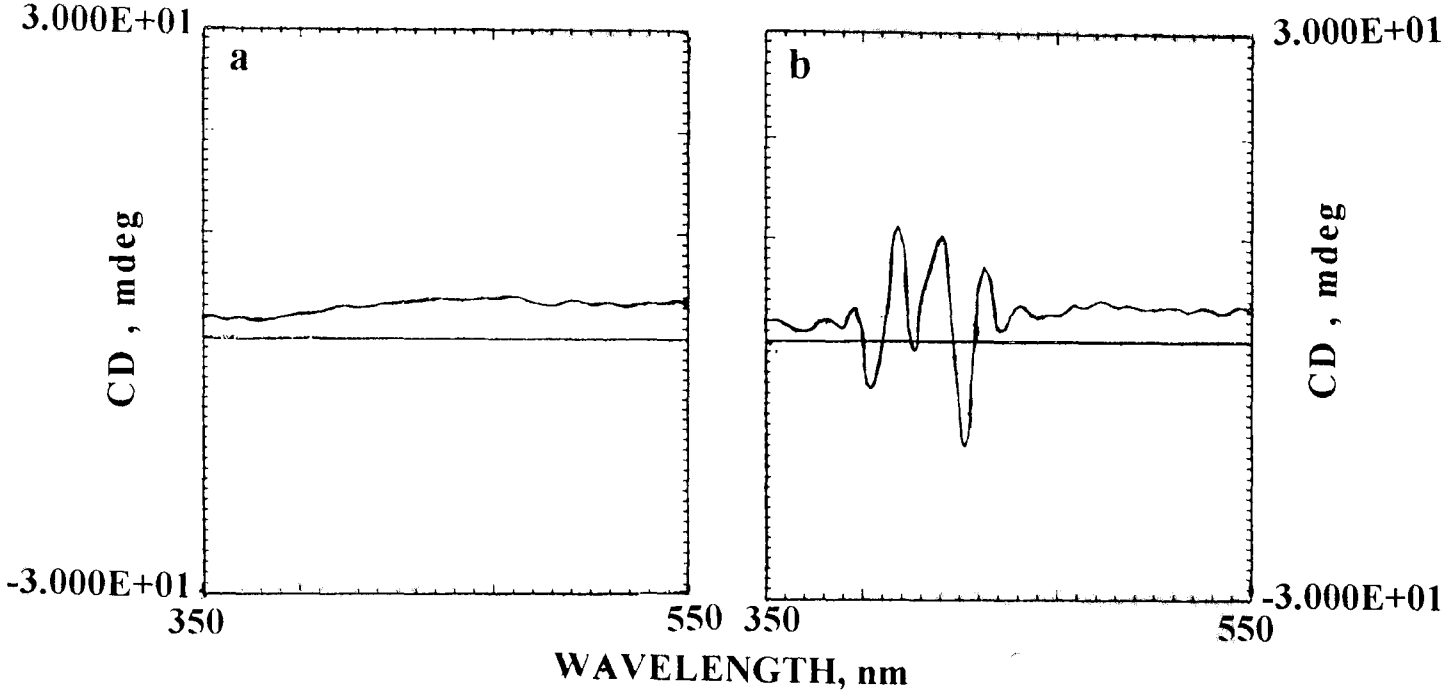


Figure 36. Visible-CD spectra of (a) free membrane and **(b)** free bilirubin in 5 mM sodium phosphate buffer, pH 8.0 containing 1 mM $MgSO_4$

wavelength transition shows a (+) long wavelength at 515 nm followed by (-) short wavelength CE at 470 nm with a shoulder at 430 nm and a small (+) short wavelength CE at 410 nm. A crossover point for major peaks is at 490 nm (Figure 37A). The appearance of major peaks (positive and negative) at these wavelengths is ascribed to the cumulative CDCE of BR interacting with a number of membrane proteins, thereby shifting the characteristic BR-protein peaks towards longer wavelength range but retaining their signs. Moreover, intercalation of BR into apolar region of membrane could alter protein-lipid interface of selected regions of membrane (Zakim & Wong, 1990).

BR complexed with human serum albumin (HSA) shows CD spectra characterized by the bisignate CDCE having minima at shorter wavelengths (405-425 nm) and maxima at longer wavelengths (400-470 nm) (Harmatz & Blauer, 1975; Lightner *et al.*, 1986; Lightner *et al.*, 1988; Blauer *et al.*, 1977). BR incubated with albumin-entrapped sealed membranes also exhibit the characteristic induced bisignate CD bands of BR-HSA complex with long wavelength transition maxima at 450 and 475 nm and minima at 415 nm with a shoulder at 390 nm complex (Figure 37C). A crossover point at 440 nm, also characteristic of BR-HSA, complex is seen. The spectrum of membrane-bound BR (Figure 37A) transformed slowly to that of HSA-bound BR (Figure 37C) suggesting that BR traverses through the membrane and is available to the HSA entrapped within the sealed membrane either from the inner side of membrane or by passing all the way along into the space within sealed membrane, interacting with albumin. A number of peaks emerge as a result of the cumulative effect of

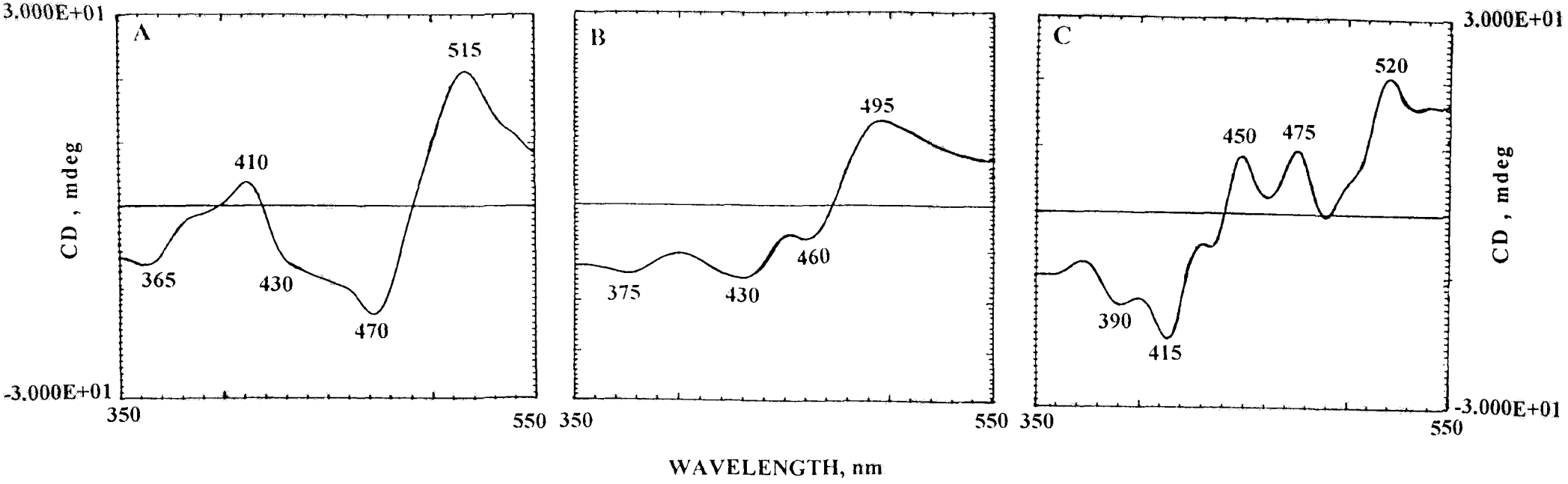


Figure 37. Visible-CD spectra of (A) bilirubin-bound sealed membrane, **(B)** bilirubin-bound HSA-adsorbed and subsequently washed sealed membrane and **(C)** bilirubin bound HSA-entrapped sealed membranes, in 5 mM sodium phosphate buffer, pH 8.0 containing 1 mM $MgSO_4$. The protein and bilirubin concentrations in each sample were 3.8 mg/3 ml of incubation mixture and 150 μM, respectively.

BR binding to HSA as well as membrane proteins. In HSA-entrapped sealed membranes the CDCE of BR-protein complex resembles more of BR-HSA complex, ascribed to the direction of BR towards the inner space where albumin is available, strengthening the idea that BR instead of remaining intercalated, is able to bypass the exterior half of the bilayer because BR binds with high affinity to albumin (Peters, 1985). Figure 37B shows the CD spectra of bilirubin binding to sealed membranes incubated with albumin and washed extensively to clear the possibility of albumin adsorption to the sealed membranes. The CD spectra, more or less, resembles that of bilirubin bound-sealed membranes with no positive peaks around 450-470 nm but negative peaks at around 430 nm and 460 nm, suggesting that albumin indeed gets entrapped within the sealed membranes. Further, their characteristic CD spectra is not because of the inefficiency of resealing resulting in the leaking of albumin from sealed membranes and its interaction with the available bilirubin to give the type of CD pattern visualized for albumin entrapped-sealed membranes but is in fact the resultant of the interaction of bilirubin with entrapped albumin implying that bilirubin traverses membrane bilayer.

The incorporation of ceftriaxone (300-1200 μM) and sodium salicylate (300-1200 μM) into the incubation mixture containing sealed membrane and BR, severely decreased the intensity of both positive and negative CDCE (Figure 38, i-iii and 38A, iv-vi). Figure 38A (i), (ii) and (iii) represent bilirubin interaction with sealed membranes after the addition of 300 μM, 900 μM and 1200 μM ceftriaxone while figure 38A (iv), (v) and (vi) represent interaction of bilirubin

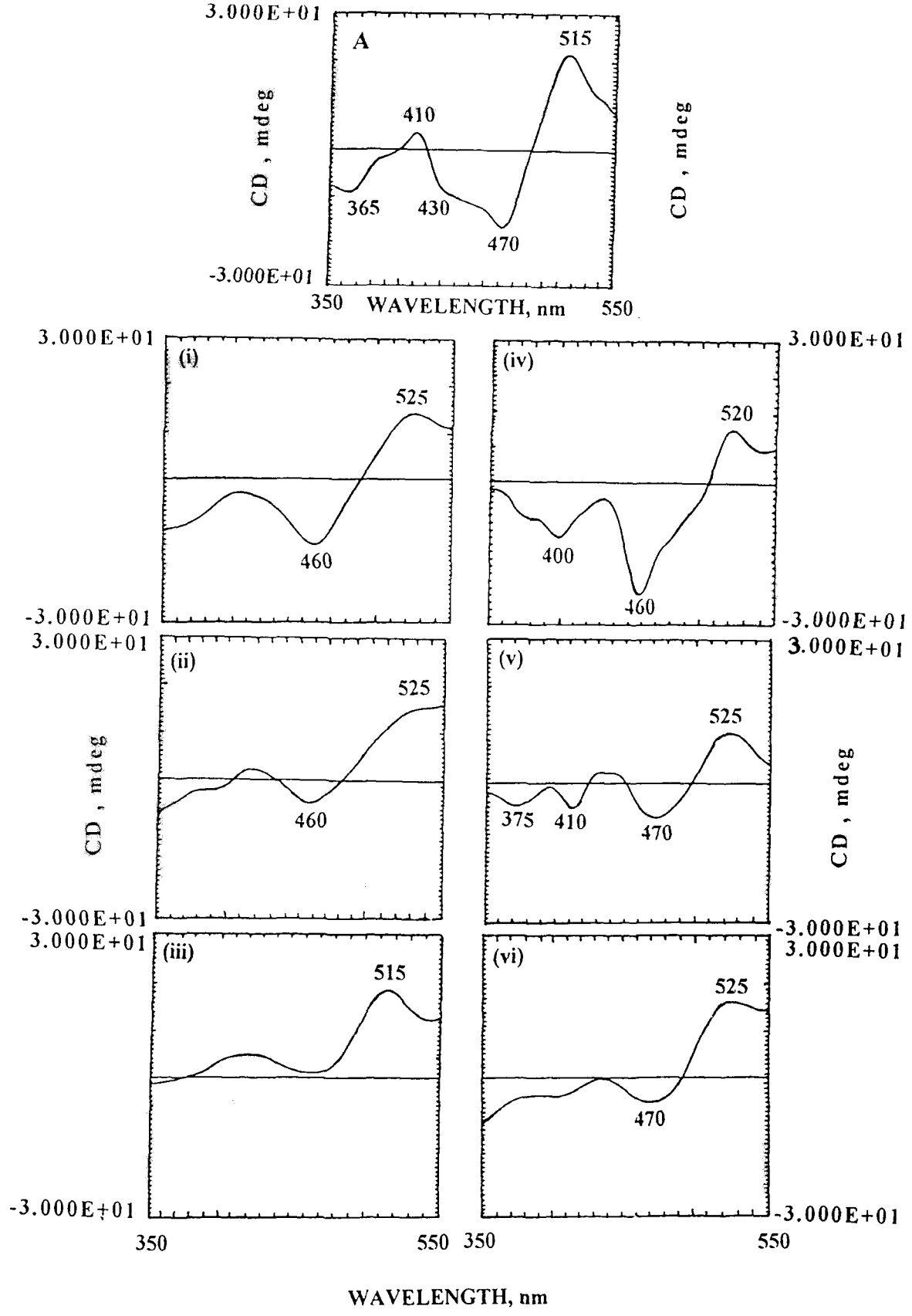


Figure 38. **(A) Visible-CD spectra of bilirubin-bound sealed membranes** incorporated with different concentrations of ceftriaxone, **(i)** 300 μM; **(ii)** 900 μM and **(iii)** 1200 μM and incorporated with different concentrations of sodium salicylate, **(iv)** 300 μM; **(v)** 900 μM and **(vi)** 1200 μM for 1 hr in 5 mM sodium phosphate buffer, pH 8.0 containing 1 mM $MgSO_4$. The protein and bilirubin concentrations in each sample were 3.8 mg/3 ml of incubation mixture and 150 μM, respectively.

with sealed membranes after addition of 300 μM, 900 μM and 1200 μM sodium salicylate. Comparing these CD spectra (Figure 38A, i-iii and 38A, iv-vi) with that of BR-sealed membranes (Figure 37A) shows that at the same concentrations of both drugs, the decrease in negative ellipticities (430-470 nm) is more significantly affected by ceftriaxone than by sodium salicylate. A considerable decrease in the magnitude of the peak at 515 nm and trough at 470 nm in sealed membranes upon addition of 1200 μM salicylate/ceftriaxone is seen (Figure 38A, iii and 38A, vi). The HSA-entrapped sealed membranes incubated with BR (Figure 37C) when incorporated with ceftriaxone (Figure 39A, i-iii) and sodium salicylate (Figure 39A, iv-vi) also exhibit significant reduction in the intensity of positive and negative CDCE, the negative ellipticities being affected more. This effect is similar to that observed earlier (Woolley & Hunter, 1970) where addition of salicylate to 1:1 BR-albumin solutions resulted in decrease in intensity of both CD bands with the trough being affected more. The positive band at around 515 nm is not affected by the incorporation of drugs, however the negative bands from 390-435 nm and the positive bands from 450-475 nm are severely diminished at a 900 μM concentration of ceftriaxone in BR-bound HSA-entrapped membranes. However, higher concentration of ceftriaxone eventually diminished the positive band at around 515 nm (Figure 39A, i-iii), which even high concentrations of sodium salicylate could not offer (Figure 39A, iv-vi). Only about 900 μM of ceftriaxone brought about significant reduction in the negative bands whereas 1200 μM of sodium salicylate was required to bring about the same extent of negative band reduction.

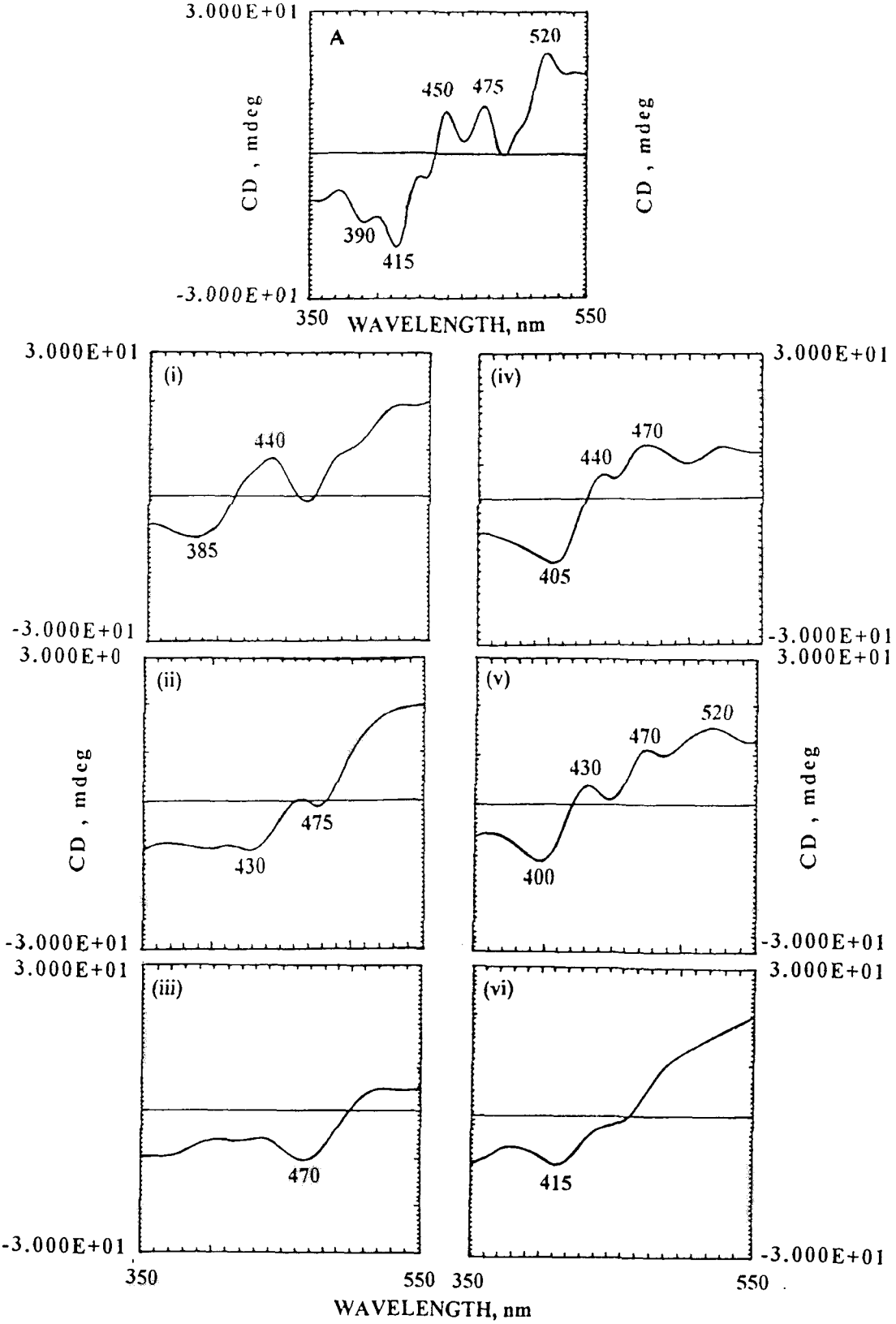


Figure 39. (A) Visible-CD spectra of bilirubin-bound HSA-entrapped sealed membranes incorporated with different concentrations of ceftriaxone, **(i)** 300 μM; **(ii)** 900 μM and **(iii)** 1200 μM and incorporated with different concentrations of sodium salicylate, **(iv)** 300 μM; **(v)** 900 μM and **(vi)** 1200 μM for 1 hr in 5 mM sodium phosphate buffer, pH 8.0 containing 1 mM $MgSO_4$. The protein and bilirubin concentrations in each sample were 3.8 mg/3 ml of incubation mixture and 150 μM, respectively.

The effect of salicylate on the binding of BR to albumin was less marked than ceftriaxone but a drug-induced BR displacement from albumin is observed in both the cases as evident from the loss of the CD bands characteristic of BR-HSA complex. This leads us to conclude that drugs bind to the same sites as that of BR-binding and can perturb the conformation of BR-protein complex. Binding of salicylate by albumin is considerably weaker than ceftriaxone as evident by the fact that the maximal displacement factor (δ) for ceftriaxone is 3.00 as compared to sodium salicylate whose δ is 1.5 (Brodersen, 1978). Thus, large doses of sodium salicylate are required to displace BR from albumin (Brodersen, 1974). Therefore, some drugs can displace BR at the same binding sites from albumin with their displacing action varying from one drug to another, also the mechanism of action on membrane may also differ for various drugs.

Here we conclude that HSA-entrapped sealed membranes exhibit CD spectrum characteristic of BR-HSA complex because HSA has much higher affinity for BR and an internal solution of HSA acts as the driving force for BR influx. BR is expected to distribute between internal albumin, within the bilayer and the surface of the lipid bilayer. Hayward *et al.* (1986) have reported that internal albumin pools exert competitive influence for BR binding to membranes and this fact necessitates the movement of BR across the liposome bilayer, quantitatively substantiating the hypothesis that BR can pass through membrane system by simple diffusion. However, partitioning of BR between albumin and membrane suggest that BR has considerable freedom of motion in membrane and not oriented by membrane. There are no groups on BR to keep the molecule at the

interface so BR partitions more exclusively into membranes. Thus, supporting the idea that BR is embedded into apolar region of membrane (Leonard *et al.*, 1989). This seems more feasible as our results with drug-induced BR displacement from albumin could not revert the BR-HSA CD signals to BR-membrane CD signals as expected if BR interacts with internal albumin. Since, this did not happen, it seems more likely that BR reaches the inner layer of the membrane bilayer and is available to albumin on the inner surface, thereby giving the CD signals characteristic of BR-HSA.

Clinical implications can be postulated as albumin is reported to enter disrupted blood-brain barrier and thus an increased entry of BR into such membrane could aggravate kernicterus in infants. Drug therapy in this case would only release the albumin-bound BR increasing free BR concentration for interaction with membranes. This would pose serious clinical complication since the effect would be a combination of BR as well as drug binding to membranes. In an attempt to reconcile these results and to further delineate the mechanism whereby BR traverses cellular membranes this simple strategy utilizing CD spectroscopy was undertaken.

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LIST OF PUBLICATIONS/PRESENTATIONS

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2. **Rashid, H.**, Ali, M.K. & Tayyab, S. (2000) "Effect of pH and temperature on the binding of bilirubin to human erythrocyte membranes"
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Proceedings of the International Symposium on Recent Trends in Biomedical Research held at TIFR, Mumbai on Sept. 27-29

The secret to success is constancy to purpose- Benjamin Disraeli

Biography

Huma Rashid was born at Aligarh, U.P. on May 6, 1974. She passed her High School Examination from I.C. High School, Benghazi, Libya in 1990. She did her Senior Secondary in 1992, B.Sc. (Hons.) Chemistry in 1995 from Aligarh Muslim University and joined for Masters in Biotechnology (1997) at Interdisciplinary Biotechnology Unit.

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Differential accessibility of bilirubin to erythrocyte membrane vesicles bearing different structural features

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Abstract

Interaction of bilirubin with different types of erythrocyte membrane vesicles such as unsealed, heterogeneous, sealed and inside-out membrane vesicles prepared from human and goat erythrocytes was studied. Out of various types of membrane vesicles, in both species, unsealed membrane vesicles bound quantitatively higher amounts of bilirubin followed by heterogeneous and sealed membrane vesicles whereas inside-out membrane vesicles bound the lowest amount of bilirubin. These differences in the amount of bound bilirubin to different membrane vesicles were correlated well with the percentage accessibility of sialic acid to neuraminidase in these membranes suggesting that bilirubin bound preferentially to the outer layer of erythrocyte membranes than the inner layer. Further, membrane vesicles prepared from human erythrocytes bound higher amounts of bilirubin than those prepared from goat erythrocytes. This can be ascribed to different phospholipid composition of these membranes.

Keywords: Bilirubin; Erythrocyte membranes; Goat; Human; Inside-out vesicles; Sealed membranes; Unsealed membranes

1. Introduction

Binding of bilirubin, a catabolic product of hemoglobin to brain cells is considered the main cause of bilirubin encephalopathy in jaundiced neonates (Hansen and Bratlid, 1986). To unravel the mechanism of entry of bilirubin into brain cells, studies have been performed on the interaction of bilirubin with biological membranes using erythrocyte membranes as a model (Sato and Kashiwamata, 1983; Sato et al., 1987). Although large number of data is available on the bilirubin–membrane interaction, no conclusion has been arrived at the mechanism of entry and localization of bilirubin in the bilayer membranes. Whereas Hayer et al. (1989) reported that erythrocytes and erythrocyte membranes having similar phospholipid content bind the same amount of bilirubin, Karp et al. (1985) on the other hand, showed more binding of bilirubin to lysed erythrocyte membranes compared with intact ghosts suggesting that bilirubin binds to both sides in lysed membranes compared with only one side binding in intact ghosts. This is supported by the work of Sato et al. (1987) who found no difference in bilirubin binding to inside-out and right-side out sealed membrane vesicles, suggesting similar distribution of bilirubin binding sites on both outer and inner surfaces of the membranes or if sites are present in the membranes, they are accessible from both sides. Contrary to this, Vazquez et al. (1988) have reported the presence of bilirubin binding sites mainly in the outer layer of bilayer membranes. In yet another study,

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Effect of pH and temperature on the binding of bilirubin to human erythrocyte membranes

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Effect of pH and temperature on the binding of bilirubin to human erythrocyte membranes was studied by incubating the membranes at different pH and temperatures and determining the bound bilirubin. At all pH values, the amount of membrane-bound bilirubin increased with the increase in bilirubin-to-albumin molar ratios (B/As), being highest at lower pH values in all cases. Further, linear increase in bound bilirubin with the increase in bilirubin concentration in the incubate was observed at a constant B/A and at all pH values. However, the slope value increased with the decrease in pH suggesting more bilirubin binding to membranes at lower pH values. Increase in bilirubin binding at lower pH can be explained on the basis of increased free bilirubin concentration as well as more conversion of bilirubin dianion to monoanion. Temperature dependence of bilirubin binding to membranes was observed within the temperature range of 7°–60°C, showing minimum binding at 27°C and 37°C which increased on either side. Increase in bilirubin binding at temperatures lower than 20°C and higher than 40°C can be ascribed to the change in membrane topography as well as bilirubin-albumin interaction.

1. Introduction

Bilirubin is a yellow pigment produced by the catabolism of haemoglobin. Under physiological conditions, bilirubin remains bound to serum albumin which carries it to the liver for further metabolism. However, when bilirubin-to-albumin molar ratio (B/A) exceeds 1 : 1, free bilirubin binds to many types of cells including brain cells which is the cause of brain toxicity in premature neonates, a condition called kernicterus or bilirubin encephalopathy. In addition, jaundiced neonates with low plasma pH have been reported to be at greater risk of developing bilirubin encephalopathy (Kim *et al* 1980). Interaction of bilirubin with cells or cell membranes is well documented (Bratlid 1972; Sato *et al* 1987; Vazquez *et al* 1988; Hayer *et al* 1989; Leonard *et al* 1989; Tayyab and Ali 1995, 1997) and it is commonly accepted that the toxicity of bilirubin depends on its passage across the plasma membrane and its association with membrane lipids (Ali and Zakim 1993; Zucker *et al* 1994). However, the way in which bilirubin interacts with biological membranes is not fully understood. Erythrocytes being simple have been commonly used to study the interaction of bilirubin with cells or cell membranes as a model system (Hayer *et al* 1989). Further, erythrocyte-bound bilirubin has been suggested as a useful criterion for the risk of bilirubin encephalopathy in neonates (Bratlid 1972). It has been reported that the interaction of bilirubin with the membranes is greatly influenced by the physico-chemical properties of the interacting media such as pH and temperature (Sato and Kashiwamata 1983). Increased binding of bilirubin to biological membranes at physiological pH (i.e., 7·0–7·2) has been suggested to be due to increased precipitation of bilirubin on the surface of membranes (Cestaro *et al* 1983). However, Sato and Kashiwamata (1983) have reported that saturable bilirubin binding to erythrocyte membranes has a pH optimum at around pH 7·1. They have suggested that the cellular susceptibility at lower pH may be determined not only by the physical state of bilirubin but also by the physico-chemical conditions of bilirubin binding substances on the membranes. Vazquez *et al* (1988) suggested that the increase in bilirubin binding to membranes at lower pH was mainly due to hydrophobic inclusion of bilirubin into membranes rather than aggregation of bilirubin on the surface of membranes. On the other hand, the influence of temperature on the binding of bilirubin is still

Keywords. Bilirubin binding; erythrocyte membranes; pH; temperature

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COMPARISON OF BILIRUBIN BINDING AND OTHER MOLECULAR PROPERTIES OF THE SERUM ALBUMIN OF SEVERAL MAMMALIAN SPECIES

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SUMMARY

In order to develop an animal model for *in vivo* testing of the displacing action of various drugs and for study of the bilirubin deposition in the brain, some molecular properties of the serum albumin from different species i.e. pig, cat, human, dog, sheep, guinea pig and rat were investigated. These albumins were found to have differences in molecular weight, Stokes radius and electrophoretic mobility. They also showed different bilirubin binding characteristics when studied by spectroscopy and fluorescence quenching. It is therefore, necessary to consider the species differences when results of animal experimentation are used as a basis for the investigation of human kernicterus.

Key Words: Serum albumin of various animals; bilirubin binding; bilirubin encephalopathy.

INTRODUCTION

Drug - induced bilirubin displacement from albumin is a factor to be considered in the development of preventive strategies against fatal kernicterus in jaundiced newborn infants (1). As a result it is suggested that all drugs for use in neonates and pregnant or lactating women should be tested for their bilirubin displacing effects (2,3). Various methods such as the horseradish peroxidase method (4) and equilibrium dialysis (5) are used in the testing of drugs on bilirubin - albumin interactions under *in vitro* conditions. Because of problems encountered in these assays such as insolubility, spectral interference or inhibition / acceleration of the reactions by some drugs, they have limited applications (6). Moreover, it is desirable to check the displacing effect of test drugs *in vivo* which is difficult to perform in newborn infants. The use of a suitable laboratory animal model would be

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Abbreviations: HSA, human serum albumin; CSA, cat serum albumin; SSA, sheep serum albumin; PSA, pig serum albumin; DSA, dog serum albumin; RSA, rat serum albumin; GPSA, guinea pig serum albumin

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